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CHARACTERIZATION OF A NON-HETEROCYSTOUS SUBTROPICAL MARINE CYANOBACTERIUM THAT PRODUCES A UNIQUE MULTICELLULAR STRUCTURE AND FACILITATES DINITROGEN FIXATION

Committee:

Jerry Brand, Supervisor

John La Claire

Mona Mehdy

Stanley Roux

Tracy Villareal

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by

Zhongkui Li, B.S.; M.S.

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Dedication

This dissertation is dedicated to my deceased parents who supported me always, and to my wife, Hong Ruan and son, Gen Li (Reagan) who have supported me and endured long absences during this study.

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Zhongkui Li, Ph.D.

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A previously un-described filamentous non-heterocystous cyanobacterium was isolated from the South China Sea. Molecular phylogenetic analyses together with morphological observations suggested that this organism should be assigned a new specific epithet. It was designated as *Leptolyngbya nodulosa* Li *et* Brand. Filaments were enclosed in a sheath, and were unusual in their flattened appearance as seen in cross section and in the frequent occurrence of a void space (as seen by transmission electron microscopy) between the trichome and the sheath. A distinctive feature of *L. nodulosa* was the presence, under low light intensities, of previously un-described multicellular structures (nodules). A *L. nodulosa* nodule consists of a portion of a filament folded and twisted into a distinct unit surrounded by a firm continuous sheath. Nodules were

highly variable in size and shape, and occurred at irregular intervals along the filament. They disappeared from filaments of cultures grown at relatively high light intensities. *L. nodulosa* cultures could be grown indefinitely in media devoid of any source of combined nitrogen. Acetylene reduction assays showed that *L. nodulosa* cultures fix dinitrogen in the dark period of a diurnal cycle under micro-oxygenic conditions. The addition of DCMU ([3-(3,4-dichlorophenyl)-1,1-dimethylurea], an inhibitor of Photosystem II) to a culture of *L. nodulosa* induced much higher rates of dinitrogenase activity and altered the cycle of activity such that most acetylene reduction occurred during the light. Measurements of dinitrogenase activity in the presence of chloramphenicol (an inhibitor of protein synthesis) indicated that dinitrogenase is synthesized in darkness and destroyed in the subsequent light period. In the presence of DCMU, a much higher dinitrogenase activity is measured, but in this case only in the light. Neither the dark-mediated dinitrogenase in the absence of DCMU nor the light-mediated activity in the presence of DCMU could be sustained for more than two days without a photoperiodic light/dark cycle. Dinitrogenase activity occurred only in non-axenic cultures of *L. nodulosa*. A single *nif*H gene, with an identical sequence in axenic and non-axenic cultures, was isolated. The requirement of heterotrophic bacteria for dinitrogenase activity in *L. nodulosa* is not yet understood.

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Abbreviations

BLAST	Basic Local Alignment Search Tool
CTAB	Cetyl trimethylammonium bromide
DAPI	4',6-diamidino-2-phenylindole
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethyl urea
DNA	Deoxyribonucleic acid
ITS	Internal transcribed spacer
LB	Luria Broth (Liquid Broth)
MASM	Modified Artificial Seawater Medium
NCBI	National Center for Biotechnology Information
N-J	Neighbor joining
OD	Optical density
rbcL	Large subunit of the ribulose-bisphosphate carboxylase gene
RCF	Relative centrifugal force
rDNA	Ribosomal deoxyribonucleic acid
SEM	Scanning electron microscope or scanning electron microscopy
SDS	Sodium dodecyl sulfate
tDNA	Transfer deoxyribonucleic acid
TEM	Transmission electron microscope or transmission electron microscopy

I. INTRODUCTION

A. General description of cyanobacteria

The cyanobacteria constitute a unique group of gram negative bacteria that were first characterized as algae due to their simple morphology and their ability to do oxygenic photosynthesis (Graham & Wilcox 2000). They are believed to be the most ancient group of oxygenic photosynthetic organisms on Earth and they played a key role in establishing oxygen in the Earth's atmosphere (Dismukes *et al*. 2000). They remain among the most significant primary producers on the Earth (Capone *et al.* 1997). The plastids in plants and algae are likely derived from a cyanobacterial ancestor (Delwiche & Palmer 1997). Cyanobacteria can be found in various aquatic and terrestrial environments, including extreme habitats such as hot springs, deserts and polar regions (Whitton & Potts 2000).

Although cyanobacteria are prokaryotes, they occur in a variety of organizational patterns, including unicells, filaments, and packets of cells embedded in mucilage (Chorus & Bartram 1999). Cells of unicellular cyanobacteria are typically spherical, cylindrical or oval. Some unicellular cyanobacteria form colonial aggregates which are surrounded by mucilage or a common sheath. Filaments represent a more complex organization. Cyanobacterial filaments can be straight or helical, but never sharply bent. Trichomes (protoplasts and cell walls of filaments, but excluding any surrounding

sheath) of some cyanobacteria glide on a solid surface, oscillate at one end while the other end is attached to a solid surface, or tremble in liquid suspension (Hoiczyk 2000). The movements of trichomes may be related to fibrils in their cell walls (Hoiczyk 1998) and are used for classification at the genus level (Anagnostidis & Komárek 1988). The filaments of most cyanobacteria are unbranched, although false branching may occur. The unbranched filaments of many species contain only undifferentiated (vegetative) cells. Other species contain specialized nitrogen-fixing cells called heterocysts distributed in a characteristic pattern along the filament. Akinetes, which are specialized resting cells capable of germinating to generate a new filament, may also distribute along the filament in a characteristic pattern (Rippka *et al.* 1979). Hormogonia are short filaments that are usually devoid of a sheath and are motile. They function in asexual reproduction, dispersal and colonization of a host (Graham & Wilcox 2000). Cells in a few genera of filamentous cyanobacteria are capable of dividing in two planes to produce true branching.

Cyanobacterial cell ultrastructure is similar to the ultrastructure of various other kinds of bacteria cells, but with the addition of thylakoid membranes that perform oxygenic photosynthesis (Van Den Hoek *et al*. 1995). The thylakoid membranes often dominate the cytoplasmic space. Prominent inclusions in many species of cyanobacteria include polyhedral bodies (carboxysomes) consisting of RUBP carboxylase, cyanophycin granules (composed of polymers of aspartate, with each aspartate linked to an arginine residue) and polyphosphate granules (a

storage form of phosphate). A few species also contain gas vesicles for buoyancy. There are at least two distinct pigmentation patterns in cyanobacteria. Most cyanobacteria have “Pattern I” pigmentation, which includes both chlorophyll a and phycobilins. Phycobilins and their associated proteins are arranged in phycobilisomes, which are typically hemispherical structures attached to the cytoplasmic face of thylakoids. A few cyanobacteria, such as *Prochloron*, *Prochlorothrix* and *Prochlorococcus* have “Pattern II” pigmentation, in which chlorophyll a and b are both present, but phycobilisomes are lacking and thylakoids are stacked in pairs (Graham & Wilcox 2000).

B. Classification of cyanobacteria (traditional approaches)

Both Bacteriological and Botanical Codes of Nomenclature are accepted for the classification of cyanobacteria. The earliest classification systems were based on the International Code of Botanical Nomenclature (Anagnostidis & Komárek 1985). Because some cyanobacetria have been named under this code and some have been named under the International Code of Bacteriological Nomenclature, the rules are not consistent and the same taxon may be described under two different names (Oren 2004).

Traditional classification of cyanobacteria is based primarily on morphological characters. The characters most often used to identify species include level of morphological organization, pigmentation, cell dimensions, cell shape, cell division planes, trichome width, cell number in a colony, and the

presence or absence of a sheath surrounding individuals or a colony (Anagnostidis & Komárek 1985). However, ecological, ecophysiological and biogeographical distribution, as well as morphological variation, ultrastructure and biochemical characteristics, along with molecular data should also be considered for meaningful classification (Komárek 2005). Unfortunately, most species are defined either by their morphological characters or by molecular data, but not by utilizing an integrated consideration of both.

Classification systems for cyanobacteria can be traced to the latter part of the 19th Century when monographs describing heterocystous cyanobacteria were prepared by Bornet and Falhaut (1888) and when Gomont (1892) studied non-heterocystous cyanobacteria. Based on fresh field-collected specimens, Geitler (1932) proposed a system which is the basis for the currently accepted traditional classification of cyanobacteria.

In a currently widely accepted system, Rippka *et al*. (1979) classified cyanobacteria into five sections, including two sections composed of unicellular cyanobacteria and three sections composed of filamentous cyanobacteria, based on morphological information derived from clones of specific strains. Table 1 shows a summary of some characters that were used to establish this system.

Table 1: Characters used for classification of cyanobacteria (modified from Rippka *et al*. 1979) correlated with traditional order names. Prochlorophytes are shown outside of the "Section" or "traditional order" classification schemes.

Section	Organization	Reproduction	Plane of Cell Division	Specialized Cells	Traditional Order Names
I	Unicellular	Binary fission or budding	One - three	None	Chroococales Pleurocapsales
II	Unicellular	Multiple fission and binary fission	One - three	None	Chroococales Pleurocapsales
III	Filamentous	Trichome breakage and hormogonia	One	None	Oscillatoriales
IV	Filamentous	Trichome breakage, hormogonia, and akinetes	One	Heterocysts and akinetes	Nostocales
V	Filamentous	Trichome breakage, hormogonia, and akinetes	More than one	Heterocysts and akinetes	Stigonematales
Not included	Prochlorophytes (contain chlorophyll b)				

Anagnostidis and Komárek (1988, 1999, 2005) proposed an alternative classification scheme which attempted to adapt a compromise system that incorporated elements of both the Bacteriological Code and the Botanical Code. However, their system is still based primarily on rules of the Botanical Code. Alternative schemes have recently been proposed by Castenholz and Waterbury (1989) and Turner (1997).

C. Molecular phylogenetic studies

DNA sequence data contain genetic information and reflect evolutionary relationships among organisms. Among DNA markers, genes coding for ribosomal RNA (rDNA) have been most widely used in phylogenetic analyses. In prokaryotes, a single rDNA operon encodes all three ribosomal structural RNAs, identified as 16s, 23s and 5s (Srivastava & Schlessinger 1990). Some prokaryotic rDNA operons also encode one or more embedded tRNAs (Boyer *et al.* 2001; Iteman *et al.* 2000). The occurrence of specific tDNA sequences within rDNA may reflect intraspecific phylogeny (García-Martínez *et al.* 1996). It has been shown that 16s rDNA is a useful tool in the study of cyanobacterial phylogeny (Giovannoni *et al*. 1988; Ishida *et al.* 2001; Nelissen *et al.* 1994; Tomitani *et al.* 2006; Tuner *et al.* 1999; Wilmotte *et al.* 1992). It appears to be useful for analysis of genera and higher levels of organization (Fox *et al.* 1992) but may not be suitable for identification of bacteria at the species level due to its slow rate of evolution (Clayton *et al.* 1995; Fox *et al.* 1992). Compared with 16s

rDNA, the internal transcribed spacer (ITS) region between 16s and 23s rDNA genes is much more variable among different strains of bacteria and may evolve more rapidly. Therefore, ITS is most suitable for phylogenetic studies at the genus and subgeneric levels. In addition to rDNA, some protein-encoding genes such as *rbc*L, *nif*H and *nif*D are useful markers for cyanobacteria phylogenetic analysis (Henson *et al.* 2004; Tomitani *et al*. 2006; Zehr *et al.* 1997).

Analyses of 16s rDNA sequences partially support the classification system of Rippka *et al*. (1979). Studies using 16s rDNA indicate that cyanobacterial Sections I, II, IV and V are each monophyletic (Giovannoni *et al.* 1988; Tuner *et al.* 1999; Litvaitis 2002). Using phylogenetic analyses based on 16s rDNA, Litvaitis (2002) provided evidence that the orders Nostocales and Stigonematales are monophyletic, the orders Chrococcales and Oscillatoriales are paraphyletic, and Prochlorophytes have three independent lineages. Zehr *et al*. (1997) analyzed *nif*H gene segments. Their results indicated that both Section IV and V are monophyletic. Although there was poor homology among *nif*H genes occurring in non-heterocystous cyanobacteria, indicating their great divergence, sequences from most heterocystous cyanobacteria formed a coherent cluster. An exception occurred in two nostocalean species which contain *nif*H sequences that diverge greatly from homologous sequences in other heterocystous cyanobacteria.

The monophyletic relationship of heterocystous taxa is also supported by the similarity of *rbc*L and 16s rDNA sequences (Tomitani *et al.* 2006). These

same authors analyzed *het*R genes in heterocystous and non-heterocystous cyanobacterial taxa. The results showed that two oscillatorialean (non-heterocystous) cyanobacteria that contain *het*R-like genes are not in the heterocystous cluster, supporting the 16s rDNA and *rbc*L results.

Although molecular data are useful in the classification of cyanobacteria, meaningful phylogeny cannot be inferred without a combination of genetic data and morphological characters, along with ecological information and physiological characteristics (Komárek 2005). Classification based on traditional characters is not always consistent with the results of molecular analyses. Based on a 16s rDNA analysis, Otsuka *et al*. (1998) reported that 15 strains assigned to 5 species of *Microcystis*, each of which has a pigment composition different from the others, may be the result of ecophenes and all of these strains might be members of the same species. Palinska *et al*. (1996) reported that 5 morphologically distinct species of *Merismopedia* may actually be members of the same species, based on > 96% similarity among their rDNA sequences.

D. The Genus *Leptolyngbya*

Traditionally, the order Oscillatoriales has been divided into genera based especially on the following characters:

- Presence or absence of a sheath
- Thickness and type of a sheath
- Gross morphological appearance of the filaments

- Occurrence of false branching
- Pigment contents
- Habitat preference

The “LPP” group (*Lyngbya*, *Phormidium* and *Plectonema*) includes a large assemblage of non-heterosystous filamentous cyanobacteria enclosed by a sheath. It is not monophyletic (Anagnostidis *et al.* 1988) and is under constant taxonomic revision (Albertano & Kováčik 1994; Unrner 1997). This group was subdivided according to the thickness of trichomes (Rippka *et al*. 1979). Those with thin filaments were designated as “Group B”, an especially problematic taxon.

Leptolyngbya was established as a genus by Anagnostidis and Komárek (1988) to include oscillatorialean species that have very thin (≤ 3 mμ diameter) trichomes consisting of cylindrical or isodiametric cells devoid of gas vacuoles, and that are enclosed in a well-defined sheath. Species designations within *Leptolyngbya*, as in other Oscillatorialian genera, are based especially on morphological characteristics of trichomes and sheaths. The characters used for identification of *Leptolyngbya* vary somewhat with environmental conditions (Anagnostidis and Komárek 1988) and the homogeneity of the genus has been questioned (Albertano 1991; Albertano and Kováčik 1992; Hoffman and Compere 1991). More recently, morphological and 16S rDNA analyses of polar and near-polar Oscillatorialeans suggested that the genus *Leptolyngbya* is not monophyletic and requires revision (Casamatta *et al.* 2005). Therefore, it has

been difficult to assign sub-generic designations to this genus (Albertano & Kováčik 1994). Although the genus *Leptolyngbya* seems problematic, an increasing number of strains have been assigned to it (Casamatta *et al.* 2005; Li & Brand 2007).

E. Dinitrogen fixation in the oceans

Biological dinitrogen fixation is the process used by living organisms to convert atmospheric dinitrogen to ammonia. Because of the stable triple bond of nitrogen gas, dinitrogen fixation is an energy demanding process. The dinitrogenase complex, dinitrogenase reductase (Fe protein) and dinitrogenase (MoFe protein), together form the central component (dinitrogenase complex) of the dinitrogen-fixing system (Postgate 1982). Dinitrogenase is a tetramer composed of two components, encoded by *nif*D and *nif*K, respectively. The dinitrogenase is reduced by dinitrogenase reductase, which is a homodimer encoded by *nif*H. The genes encoding these three components of the dinitrogenase complex always occur in the same operon, and are designated as *nif*HDK. The organization of these genes is very similar in heterotrophic bacteria and non-hetereocystous cyanobacteria that fix dinitrogen, but is slightly different in some heterocystous cyanobacteria (Bergman *et al.* 1997). Some dinitrogen fixing organisms also have genes that encode molybdenum-independent dinitrogenase (Pau 1991; Smith and Eady 1992), although none has been reported in non-heterocystous cyanobacteria . Two different molybdenum

dinitrogenase complexes have been found in *Anabaena variabillis*. One is expressed only in heterocysts and functions in filaments under both aerobic and anoxic conditions. The other is expressed in both vegetative cells and heterocysts, but only in filaments that are under anoxic conditions (Schrautemeier *et al.* 1995; Thiel *et al.* 1995). The synthesis and expression of dinitrogenase are determined by various physiological and ecological factors, including the presence of oxygen, the presence of a combined nitrogen source, the availability of iron and molybdenum ions, temperature and an adequate energy supply (Karl *et al.* 2002).

Dinitrogen fixation is an important component of the global nitrogen cycle. Primary production in tropical and subtropical oceans is believed to be limited by nitrogen availability (Falkowski 1997). Combined nitrogen may become available through dinitrogen fixation by diazotrophs and/or by an upward vertical flux of nitrate from deep waters (Lewis *et al.* 1986). Older surveys indicated that marine dinitrogen-fixing organisms were rare and dinitrogen fixation was of minor importance in marine pelagic environments (Capone & Carpenter 1982; Lipschultz & Owen 1996). Recent studies suggest that biological dinitrogen fixation is much more important than previously reported (Gruber and Sarmiento 1997; Karl *et al.* 2002). It is now believed that dinitrogen fixation in tropical and subtropical oceans may have a major role in the global marine nitrogen budget (Gruber *et al.* 2002; Karl et al. 2002). For example, in the subtropical North Pacific Ocean, dinitrogen fixed by cyanobacteria may fuel up to half of new

production (steady-state production of organic matter available for export) (Karl *et al.* 1997). Dinitrogen fixation by the microbial community in the subtropical Atlantic and Pacific oceans may account for up to 58% of the total nitrogen demand of these waters (Carpenter *et al.* 1999; Dore *et al.* 2002).

All diazotrophs are prokaryotic, but only a small portion of all known prokaryotes can fix atmospheric dinitrogen. Diazotrophs are widely distributed in the oceans, and are taxonomically, physiologically and ecologically diverse. These organisms include members of heterotrophic bacteria, cyanobacteria and archaea (Karl *et al.* 2002). Dinitrogen fixation in cyanobacteria is more complex than in heterotrophic bacteria because cyanobacteria produce molecular oxygen in the process of photosynthesis. All major cyanobacterial groups include members that have been reported to fix nitrogen (Bergman *et al.* 1997). Section IV and V cyanobacteria in the system of Rippka *et al*. (1979) develop specialized cells (heterocysts) for dinitrogen fixation. Heterocyst cells have thick cell walls and contain unpaired thylakoids that are incapable of Photosystem II activity. The oxygen concentration in heterocysts is low since it can diffuse only slowly through the thick cell walls, it is not evolved photosynthetically, and it is consumed in respiratory processes (Murry *et al*. 1984). Dinitrogenase can be expressed and become active under an oxic condition within heterocysts. Oxygenic photosynthesis and dinitrogen fixation are separated physically in heterocystous cyanobacteria since only vegetative cells evolve oxygen photosynthetically, while only heterocysts perform dinitrogen fixation under

aerobic conditions. Cytoplasmic connections between heterocysts and vegetative cells facilitate the transfer of reduced nitrogen from heterocysts to vegetative cells and the transfer of reduced carbon compounds from vegetative cells to heterocysts (Jüttner 1983).

Dinitrogen-fixing non-heterocystous cyanobacteria are different from heterocystous cyanobacteria in that they appear to fix dinitrogen in the same kinds of cells that perform photosynthesis. Species of *Plectonema*, *Phormidium* and *Lyngbya* fix dinitrogen only in darkness because oxygen produced during photosynthesis inactivates dinitrogenase in the light. However, non-heterocystous *Trichodesmium* is unique in that it fixes dinitrogen only in the light. *Trichodesmium* is widely distributed in tropical and subtropical oceans (Capone *et al.* 1997). There are two forms of *Trichodesmium* aggregates. Trichomes in spherical aggregates (puffs) radiate from a central area and are imbedded in a firm mucopolysaccharide matrix. Trichomes in the second form (tufts) occur in a parallel loosely-aggregated bundle. Colonies can change their structure from tufts to puffs. The mechanism used by *Trichodesmium* to separate oxygen produced in photosynthesis from dinitrogenase is enigmatic. An early widely-accepted hypothesis was that dinitrogen fixation in *Trichodesmium* is confined to the central region of both puffs and tufts, where oxygen is consumed and the rate of photosynthesis is low (Bryceson & Fay 1981; Carpenter & Price 1976). Recent evidence suggests that both spatial and temporal segregation of dinitrogen fixation and photosynthesis occur in *Trichodesmium* (Berman-Frank *et al.* 2001).

Transmission electronic microscopy of immunogold-label filaments indicated that dinitrogenase occurs only in certain cells along a filament, thereby spatially separating photosynthesis from dinitrogen fixation (Janson *et al.* 1994). This separation model has been supported by florescence whole-cell immunolocalization observations, which identified dinitrogenase in only 10 - 15% of the cells along a filament (Lin *et al.* 1999). Dinitrogen fixation has been much more extensively studied in *Trichodesmium* than in any other oceanic organism. Although *Trichodesmium* is often proposed to account for the vast majority of dinitrogen fixation in tropical and subtropical oceans (Capone *et al.* 1997), recent measurements and calculations of nitrogen budgets indicate that a variety of other diazotrophs may also be important for marine dinitrogen fixation (Zehr *et al.* 1998, 2001). A number of unicellular cyanobacteria and proteobacteria ranging in size from 0.2 to 10 μm in the subtropical North Pacific Ocean were demonstrated to carry *nifH* gene fragments, with cyanobacteria predominating (Zehr *et al.* 2001). Organisms of this same size range, from approximately the same geographical locations, were found to fix dinitrogen at high rates (Montoya *et al.* 2004). Analyses of *nifH* gene fragments from planktonic organisms in the tropical and subtropical North Atlantic Ocean indicated that there is a vertical community stratification of diazotrophs, with filamentous cyanobacteria (predominantly *Trichodesmium*) most prevalent in surface waters, and unicellular cyanobacteria and heterotrophic proteobacteria dominant in deeper waters (Langlois *et al.* 2005).

Heterotrophic bacteria that can fix dinitrogen in marine environments include Eubacterial aerobes, microaerophiles, facultative anaerobes and obligate anaerobes, as well as Archaea (Herbert 1999). Dinitrogen fixation by unicellular bacterioplankton may be almost as great as in *Trichodesmium* spp. in the tropical North Atlantic, although less dinitrogenase activity by bacterioplankton was measured in the subtropical North Pacific (Falcón *et al.* 2004). In near-surface waters heterotrophic proteobacteria *nif*H transcripts were identified during both night and day, while unicellular cyanobacterial *nif*H transcripts were present only during the night (Falcón *et al.* 2004). For these heterotrophic bacteria to fix nitrogen, a reduced substrate must be available to provide energy and reductant to the dinitrogenase complex, and a means of protecting dinitrogenase from oxygen inactivation must be provided (Zehr *et al.* 2001).

Symbiotic associations of dinitrogen-fixing and non-diazotrophic organisms occur widely in nature (Carpenter 2002). *Richelia* is a heterocystous cyanobacterium that lives in a symbiotic relationship with the diatoms *Rhizosolenia* and *Hemiaulus* in pelagic marine waters (Villareal 1987, 1991). Dinitrogenase genes derived from heterotrophic bacteria that are associated with diatom mats have also been recovered (Zehr *et al.* 2001).

An increasing number of diazotrophs have been found to play important roles in dinitrogen fixation and nitrogen cycling in warm marine environments. In this dissertation, I describe a strain of non-heterocystous cyanobacterium

isolated from the South China Sea that produces a previously undescribed multicellular structure and which is capable of facilitating dinitrogen fixation.

II. MATERIALS AND METHODS

A. General maintenance and measurements of *L. nodulosa*

Cultures of *L. nodulosa* were maintained in 250-mL flasks or in 30-mL test tubes in MASM or MASM-0 medium (Appendix I) under a diurnal cycle of 12 h light:12 h dark at 25°C without disturbance (no mechanical agitation or bubbling). Agar cultures were maintained on slants under the same conditions in 1.5% agar. For growth of *L. nodulosa* devoid of nodules, liquid cultures were placed under a light intensity of 60 µmol photons m^{-2} sec^{-1}. For formation of nodules, cultures were transferred from a high light intensity to an intensity of ~15 µmol photons m^{-2} sec^{-1}. Cultures maintained in medium without combined nitrogen were kept at a light intensity of 6 - 15 µmol photons m^{-2} sec^{-1}.

1. Measurement of growth rates: A flask containing a culture was vigorously agitated to distribute the culture uniformly within the liquid. The suspension was homogenized with a hand-held tissue grinder equipped with a Teflon pestle. This process disrupted filaments into short fragments containing generally <10 cells/filament. No detectible phycobilins were released from the homogenized cultures and the short filaments remained viable. Two-mL volumes of the homogenized culture were distributed into 25-mL flasks. MASM was added to each flask to a total volume of 10 mL. At 2-day internals the total amount of culture of one of the flasks was measured. Two separate kinds of measurements

were used to determine the amount of culture: packed cell volume and chlorophyll concentration. The increase in packed cell volume or chlorophyll concentration in successive 25-mL flasks as a function of time determined the growth rate of the culture.

2. Measurement of packed cell volume: A 4-mL volume of homogenized culture was placed into a calibrated glass centrifuge tube that measured volumes at the bottom of the tube in 1 µL increments. The centrifuge tube was subjected to centrifugation (RCF 1,000xg for 10 minutes) to pellet the short filaments into the calibrated bottom of the tube, leaving a clear supernatant. The volume of the pellet was estimated to the nearest 0.5 µL. Packed cell volume was determined as [volume of pellet]/[total volume in the centrifuge tube].

3. Measurement of chlorophyll concentration: A known volume of culture was subjected to centrifugation (RCF 1,000xg for 10 minutes) and the pellet was suspended in a known volume of 80% acetone. The acetone suspension was dispersed with a glass homogenizer equipped with a Teflon pestle, and then heated to 60°C for 5 minutes. The heated extract was subjected to centrifugation (RCF 1,000xg for 5 minutes) to remove particulate matter and the clear green supernatant was transferred to a clean test tube. Optical density of the contents of the tube was measured at 663 nm and the chlorophyll

concentration was determined from the extinction coefficient at 663 nm (Arnon 1949).

4. Measurement of wet weight: An aliquot of culture grown in MASM-0 was removed with a forceps, gently blotted with a laboratory wiper, placed on waxed paper and finally weighed to the nearest 0.01 gram. It was then immediately transferred to a 25 mL assay flask for measurement of acetylene reduction.

5. Measurement of oxygen evolution: Measurements of changes in oxygen concentration vs in light or darkness were measured with an oxygen monitor (YSI 53) equipped with a Clark-type electrode and recorded with a strip-chart recorder. Temperature in the 1.4-mL continuously stirred chamber was maintained at 25°C for all measurements. The intensity of the unidirectional light source (tungsten-halide lamp) was controlled with neutral density filters and measured with a Li-Cor Model LI-185A Quantum photometer equipped with a Li-Cor Model 3472 sensor. All oxygen concentration measurements were of cultures in MASM.

6. Measurement of absorption spectrum: Prior to measuring its absorption spectrum, a culture was homogenized for ~30 seconds with a glass homogenizer equipped with a Teflon pestle to disrupt clumps and produce

filaments containing generally less than 10 cells each. The resulting homogenous suspension was transferred to a rectangular glass cuvette (1 cm path length) into which a 3 mm Lucite scattering plate had been inserted to correct for sample scattering. The absorption spectrum of the sample was measured with respect to a blank cuvette equipped with the same Lucite scattering plate, using a Beckman DU500 spectrophotometer. Spectral data at 1 nm resolution were recorded and stored in a computer.

7. Preparation of axenic cultures: Cultures were rendered axenic by a heat-shock and washing method. A 20-mL test tube containing 5 mL of culture was heated to 45°C for 5 minutes. The tube containing heat-shocked culture was inserted into a sonic bath (Branson Model 2510) for 30-60 seconds, until the suspension appeared to be uniform. The culture was checked microscopically to confirm that nearly all filaments contained less than 10 cells. The sonicated culture was subjected to centrifugation at an RCF of 300xg for 5 minutes at room temperature. The supernatant was discarded and the pellet was suspended in fresh culture medium. This same washing procedure was repeated two more times. The final pellet was suspended in 0.5 mL of MASM medium. The suspension was streaked on agar plates containing MASM in 1.5% agar. Plates were placed in darkness overnight, and then transferred to dim light (less than 15 μmol photons m^{-2} sec^{-1}, 12 h light:12 h dark) for approximately a week. Plates were then transferred to bright light (~60 μmol photons m^{-2} sec^{-1}, 12 h light:12 h

dark) and left undisturbed until colonies were visible. Individual colonies were transferred to fresh MASM agar slants.

Putative axenic cultures were superficially checked for bacterial contamination by microscopic examination, using DAPI-stained (see below) cells. For more critical determination of bacterial contamination, a portion of an agar slant was aseptically transferred into a 50-mL flask containing 20 mL of sterile liquid MASM-LB medium (MASM that also contained the components of LB medium). The culture was incubated in darkness at 25°C for at least two weeks. Media that contained contaminated cultures appeared cloudy and revealed a high concentration of heterotrophic bacteria when observed microscopically, while media containing axenic cultures remained clear and devoid of microscopically visible bacteria.

B. Microscopy

1. Light and confocal microscopy: Wet mounts of filaments in culture medium were observed using bright-field, fluorescence and DIC Nomarski optics with an Olympus BX-60 compound optical microscope equipped with an Olympus DP70 digital camera. For observing DAPI florescence, a drop of culture was combined with a drop of DAPI (1 µg/mL in distilled water) on a glass slide prior to the addition of a cover slide. DAPI and chlorophyll fluorescence were observed simultaneously using a U-MWU filter set.

Confocal images were observed with a Leica SP2 AOBS microscope equipped with a 63X oil-immersion lens. The microscope was connected to a computer equipped with Leica Confocal Scanning (LCS) software. Visible light at 594 nm was used for excitation. Fluorescence emission was collected between 605 nm and 700nm. A Z-series scan was used to capture images, which were then constructed into a maximum intensity projection image. A series of scans at different optical depths were recorded and assembled to construct a 3-D image.

2. Scanning electron microscopy: Samples were fixed for scanning electron microscopy by incubating a concentrated suspension of filaments in MASM with 2.5% glutaraldehyde for 2 hours at room temperature, then washing three times in 0.05 M HEPES buffer (pH 7.2) by centrifugation at an RCF of 500xg for 5 minutes. The final pellet was dehydrated by successive 1-hour immersions in 25%, 50%, 75% and 100% ethanol, with centrifugation to pellet the sample between each immersion. Dehydrated filaments were placed on holders, coated with 8 nm gold beads, and observed with a Philips 515 scanning electron microscope.

3. Transmission electron microscopy: Two different methods were used to prepare samples for transmission electron microscopy.

Method I: A concentrated suspension of filaments was fixed with a solution of 1% paraformaldehyde plus 1% gluteraldehyde in 0.05 M HEPES buffer (pH

7.2) for 1.5 hours. The fixed sample was washed three times in HEPES buffer by centrifugation (500xg for 5 minutes), fixed for three hours with 2% OsO_4 in HEPES buffer, then washed three times with HEPES buffer by centrifugation. Fixed samples were dehydrated by 1-hour immersions in successive concentrations of 25%, 50%, 75% and 100% ethanol. Dehydrated samples were embedded in Spurr's epoxy resin and allowed to harden overnight. Thin sections (60 - 90 nm thickness) of the specimen were cut and placed on a copper grid. Sections were post-stained with 2% (w/v) uranyl acetate and 4% (w/v) lead citrate, in preparation for transmission electron microscopic observations.

Method II: A concentrated suspension of filaments was frozen in liquid propane and left undisturbed at -150 ºC for 48 hours. The frozen sample was then dehydrated with successive concentrations of methanol (50%, 75%, 100%) at -45ºC. The dehydrated sample was embedded with Lowicryl HM20 and polymerized by exposure to UV light at -45ºC for 48 hours, then allowed to warm to ambient temperature. Thin sections (60 - 90 nm thickness) of specimen were prepared and placed on a copper grid, in preparation for transmission electron microscopic observations. Samples were not post-stained.

Samples prepared by Method I or Method II were observed and photographed using a Philips EM208 transmission electron microscope.

C. Preparation of DNA and phylogenetic analysis

1. Extraction of DNA: DNA was extracted using a CTAB-SDS method. Lysis Buffer contained 3% CTAB, 1% SDS, 100 mM Tris pH 8.0, 100mM EDTA, 1.4 M NaCl and 20 µL Proteinase K (10 mg/ml/L). The protocol for extraction was as follows

a. Collect ~ 0.2 g (wet weight) of algae by centrifugation (500xg for 5 minutes); wash by centrifugation 3 times in distilled water.
b. Transfer the washed pellet to a small ceramic mortar with a spatula; add ~0.1 g of glass beads (0.12 mm diameter) to the mortar and grind for 1-2 minutes in order to break the cells.
c. Add 500 µL Lysis Buffer to the mortar and transfer the suspension to a 1.5-mL microtube; incubate the microtube in a water bath at 55°C for 30-40 minutes.
d. Add 500 µL of a mixture of phenol:chloroform:isoamyl alcohol (25:24:1) to the microtube. Mix thoroughly, then incubate for 15-30 minutes at room temperature or until the top layer is well separated from the rest of the solution. Centrifuge at an RCF of 7,000xg for 10 minutes.
e. Transfer the upper phase of the supernatant to a clean microtube and repeat step d. After centrifugation transfer the upper phase of the supernatant to a clean microtube and add 500 µL of chloroform; mix thoroughly. Centrifuge at 7,000xg for 10 minutes.

f. Transfer the upper phase of the supernatant to a clean microtube and measure the volume of solution (designated here as *V*) with a pipette. Add 1/10 *V* of 3 M sodium acetate (pH5.2) and 2 *V* of 100% ethanol to the mixture. Incubate the solution at 4°C for 1 hour.

g. Centrifuge at 13,000xg for 10 minutes. Discard the supernatant. Rinse the pellet with 70% ethanol; leave set at room temperature for 5 - 10 minutes to dry.

h. Add 100 µL distilled water to dissolve the DNA. Measure the OD_{260} and OD_{280} with a Nanodrop ND-1000 spectrophotometer.

The quantity of DNA in each sample was determined by its OD_{260} measurement, and its purity was determined by the OD_{260}/OD_{280} ratio.

2. Amplification and sequencing: Amplification of 16S rDNA was according to Svenning *et al.* (2005). The procedure for amplification of ITS was from the method of Boyer *et al*. (2001) modified by using primer 1 and 5 to get ITS region directly rather than using other primers to first produce a long fragment. The primers used for amplification of 16s rDNA and ITS are shown in Appendix IV.

For amplification of a portion of the *nif*H gene, a pair of degenerate primers (Appendix IV) was synthesized according to Zehr *et al*. (1989). Amplification was performed by first heating the sample to 95ºC for 5 minutes, performing 5 cycles of {90ºC for 1 minute, 48ºC for 1 minute and 72ºC for 1

minute}, then performing 25 cycles of {90ºC for 1 minute, 52ºC for 1 minute and 72ºC for 1 minute}, and finally leaving the sample at 72ºC for 10 minutes before it was removed from the thermocycler.

All PCR reactions were performed using the Takara DNA polymerase kit R001 on a GeneAmp PCR System 2700. The size and purity of the PCR products were determined by electrophoresis on a 0.7% agarose gel, then staining with ethidium bromide and detected with a UV trans-illuminator. PCR products were purified using a Sigma GenElute PCR Clean-up Kit. The amplified products were stored at 4ºC.

The purified PCR products were sequenced with a Perkin-Elmer ABI Prisom^{TM} 377 sequencer, using the same primers as were used for amplification. BLAST (National Center for Biotechnology Information) searches were performed to determine closest matches. ClustalX software was used to make primary alignments and Bioedit (Ibis Therapeutics, CA, USA) software was used to improve the alignments. Phylip 3.65 software (Felsenstein 1989) was used to generate Maximum Likelihood and Neighbor-Joining trees.

3. Cloning of the *nif*H gene fragment into a plasmid: The amplified *nif*H fragment was cloned into a plasmid using the TOPO DNA Cloning Kit (Invitrogen). Transformation and screening of *E. coli* was performed according to instructions that accompanied the kit. Screened colonies were grown in LB liquid medium with the addition of ampicillin (80 µg/mL). Plasmids were isolated using a

QIAgenprep Spin Miniprep Kit (QIAgen Sciences) according to the instructions that accompanied the kit. Plasmids from 5 colonies were sequenced using the same primers as were used for amplification of the *nif*H gene fragment.

D. Acetylene reduction assays

An aggregate of *L. nodulosa* was removed from its growing culture, gently blotted with a laboratory wiper, and placed on a piece of waxed weighing paper. The aggregates were weighed and then transferred to a 25-mL flask containing 5.0 mL of MASM-0. The total volume of the liquid suspension in the flask was determined by subtracting the combined weight of the MASM-0 and culture aggregate from total weight of the flask containing the suspension. A serum cap was placed over the flask to prevent equilibration with ambient air, providing a total volume in the flask of 25.0 mL. Additions to the flask were made with a hypodermic needle. In preparation for gas chromatographic analysis, the flask was flushed with pure dinitrogen gas. Acetylene was prepared by exposing calcium carbide to water and trapping the gaseous product. A 4.0-mL volume of pure acetylene was then injected into the flask. In most experiments a solution of $NaHCO_3$ was also injected into the flask to a final concentration of 0.05 M. Other reagents were added to the flask as required in individual experiments, the flask was then incubated by gentle agitation (60 cycles/minute) at 25°C (Innova 4340).

Gas chromatography was performed on an Agilent 6890N Network GC System equipped with an AT-Alumina column (30 m x 0.53 mm, Alltech) and a

flame ionization detector. Ethylene in a flask was detected by transferring 100 µL of the gas phase above an incubated culture to the GC. The GC was programmed such that immediately after injection the column was held at 80ºC for 5 minutes, then increased in temperature at 25ºC/minute to 180ºC, and finally was held at 180°C for 5 minutes. The retention times of acetylene and ethylene (precision among replicates < 4%) were determined with reference gases. Standard ethylene gas (1,000 ppm concentration, Matheson) was used for calibration and normalization. The concentrations of both acetylene and ethylene were calculated and stored on a computer. Calculation of rates of ethylene production (dinitrogen fixation activity) rates is described in Appendix V.

E. Deposit of the strain to UTEX Culture Collection of Algae and deposit of gene sequences into GenBank

A living culture of *Leptolyngbya nodulosa* Li *et* Brand has been deposited in the Culture Collection of Algae at the University of Texas at Austin (accession number UTEX 2910).

Complete ITS (508 bp) and partial 16S rDNA (1383 bp) sequences have been deposited in GenBank (Accession Numbers EF122599 and EF122600, respectively).

III. RESULTS

A. Collection and general observations of *Leptolyngbya nodulosa*

1. Collection

Water samples were collected from the South China Sea near Huizhou City in Guangdong Province (estimated coordinates 114° 16' E, 22° 70' N) at a depth of approximately 10 m with a 30 μm tow net on March 5th, 1998. The water temperature was approximately 25°C. No analysis of water quality or measurements of opacity were performed. The samples were placed into 250-mL plastic bottles, then examined with a bright field microscope. Pennate diatoms were observed, but no cyanobacteria were seen. Several hours after collection, the water samples were diluted in a series of tubes containing f/2 medium (Guillard & Ryther 1962). No color was seen in any of the tubes at the time of dilution. The tubes were maintained at ambient temperature under dim room light, and were periodically observed microscopically. After 5 months a blue-green color was seen in several tubes of the dilution series. A filamentous cyanoabcterium dominated the colored cultures, although a high density of pennate diatoms and a flagellated protozoan were also observed. The unique feature of the cyanobacterium was the appearance of knot-like structures of various sizes and shapes at irregular intervals along the filaments. Many of the protozoa (tentatively identified as *Bodo*) were attached along the surface of the

cyanobacterial filaments. Cultures were rendered unialgal and all visible protozoa were removed by capturing individual filaments with a capillary pipette in diluted culture while observing under a microscope. The unialgal cultures were then maintained in f/2 medium until they were shipped to the Culture Collection of Algae at the University of Texas at Austin (UTEX) in January, 2003. Later analysis of the strain led to the assignment of a new species name, *Leptolyngbya nodulosa*. This name will be used throughout the dissertation. The knot-like structures in filaments of *L. nodulosa* grown at low light intensity are now called nodules.

2. Axenic cultures and maintenance

Cultures were rendered axenic using a heat shock and washing method (Fig. 1).

L. nodulosa could be maintained on solid media (MASM in 1.5% agar), but liquid cultures grew much more rapidly. Growth rates were examined in several liquid media at various temperatures. Optimal growth rates (doubling time ~3.5 days, Fig. 2) were obtained in MASM liquid suspension cultures at 25°C under diurnal (12 h on: 12 h off) cool-white fluorescence light at a unidirectional intensity of 60 µmol photons m^{-2} sec^{-1}. Under these conditions, undisturbed cultures appeared as blue-green macroscopic loose aggregates floating on the surface or settled to the bottom of the glass container, but not adhering to any surface (see Fig. 18).

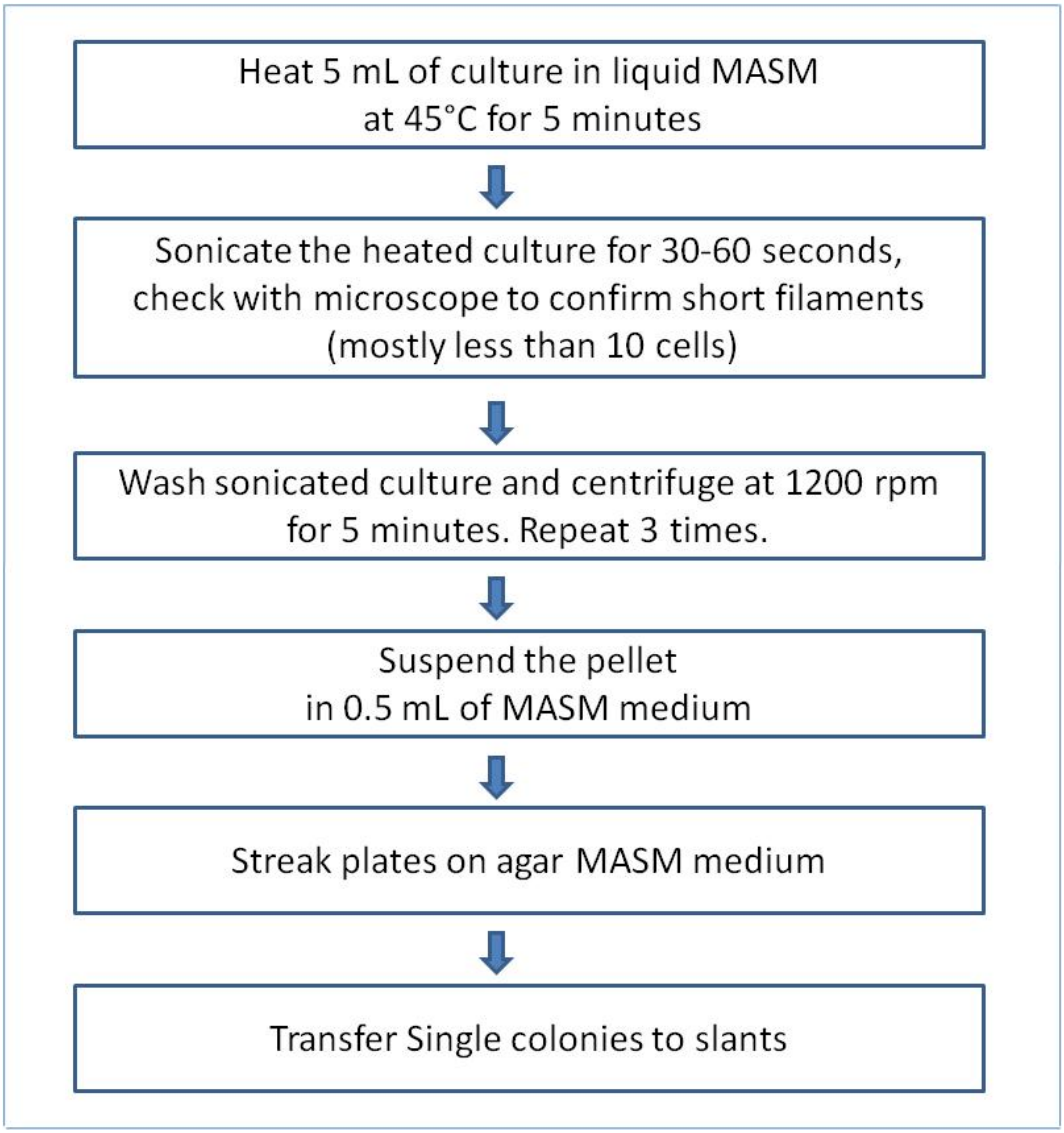

Fig. 1: Flow chart of steps in the preparation of axenic cultures of *L. nodulosa.* See Materials and Methods for details.

Slants of axenic *L. nodulosa* were checked for bacterial contamination with Liquid Broth medium, and for fungal contamination with Potato-Dextose media. Tests of colony formation on agar plates were not reliable because some

kinds of bacteria that grow in liquid media did not grow on agar. Cultures in MASM continued to grow slowly at a very low light intensity (6 µmol photons m^{-2} sec^{-1}). They remained viable and retained their deep blue-green color for at least a year in complete darkness, but did not show any detectable growth in the absence of light.

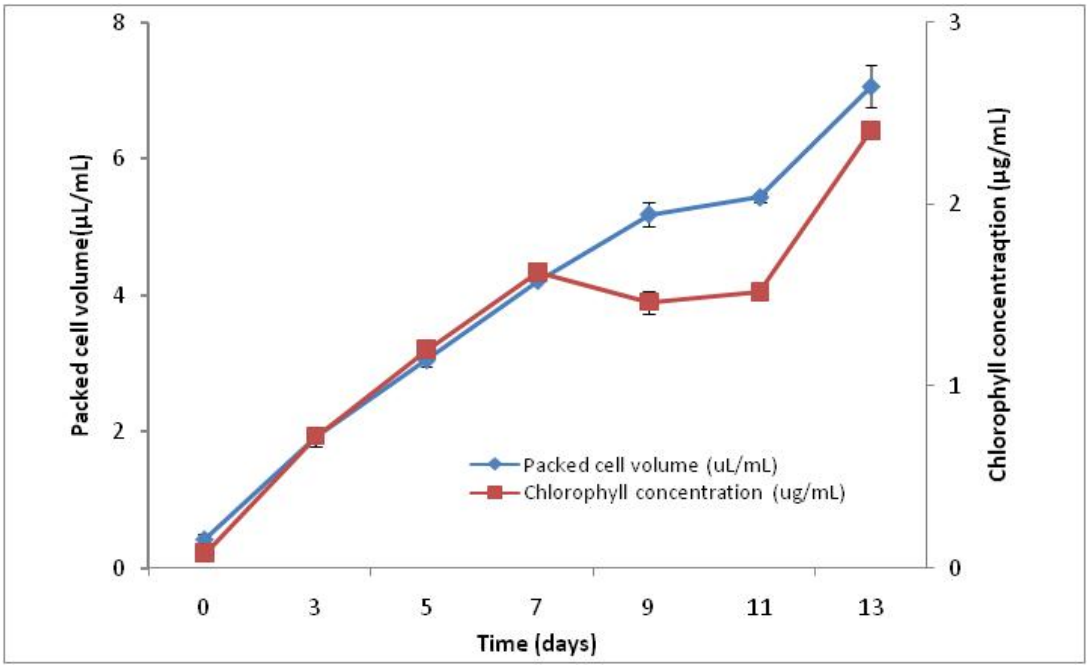

Fig. 2: Growth rates of *L. nodulosa* based on OD_{663} and packed cell volume in a 10-mL culture. The culture was grown at 25°C at light intensity of 60 µmol photons m^{-2} sec^{-1}. Procedures for preparing a homogenous culture and obtaining measurements are described in Materials and Methods. Bars indicate the average of two inocula in separate growing cultures.

3. Pigment composition of *L. nodulosa*

The color of *L. nodulosa* cultures is influenced greatly by culture conditions. For example, cultures grown at high light intensity are somewhat yellow-green in color, while cultures grown at low light intensity appear deep blue-green. Absorption spectra (600 – 700 nm) of whole filaments of *L. nodulosa* in culture media with or without a source of combined nitrogen are shown in Fig. 3. Cultures grown in the presence of combined nitrogen have a pronounced peak at 620 - 625 nm in addition to the chlorophyll absorption peak at 680 nm, demonstrating the presence of phycocyanin. In contrast, cultures grown without a source of combined nitrogen are yellow green in color and displays much lower absorbance at 620-625 nm relatively to chlorophyll, indicating a greatly reduced amount of phycocyanin.

The absorption spectrum of a lipid extract of *L. nodulosa* dissolved in 80% acetone is shown in Fig. 5. Peaks at 430, 620 and 665 nm correspond to absorbance maxima of chlorophyll a. The peak at 490 nm corresponds to carotenoid absorbance. No absorbance peak near 645 nm was observed, demonstrating the absence of detectable chlorophyll b. Thus, *L. nodulosa* has a pigmentation pattern characteristic of cyanobacteria; it is not a prochlorophyte.

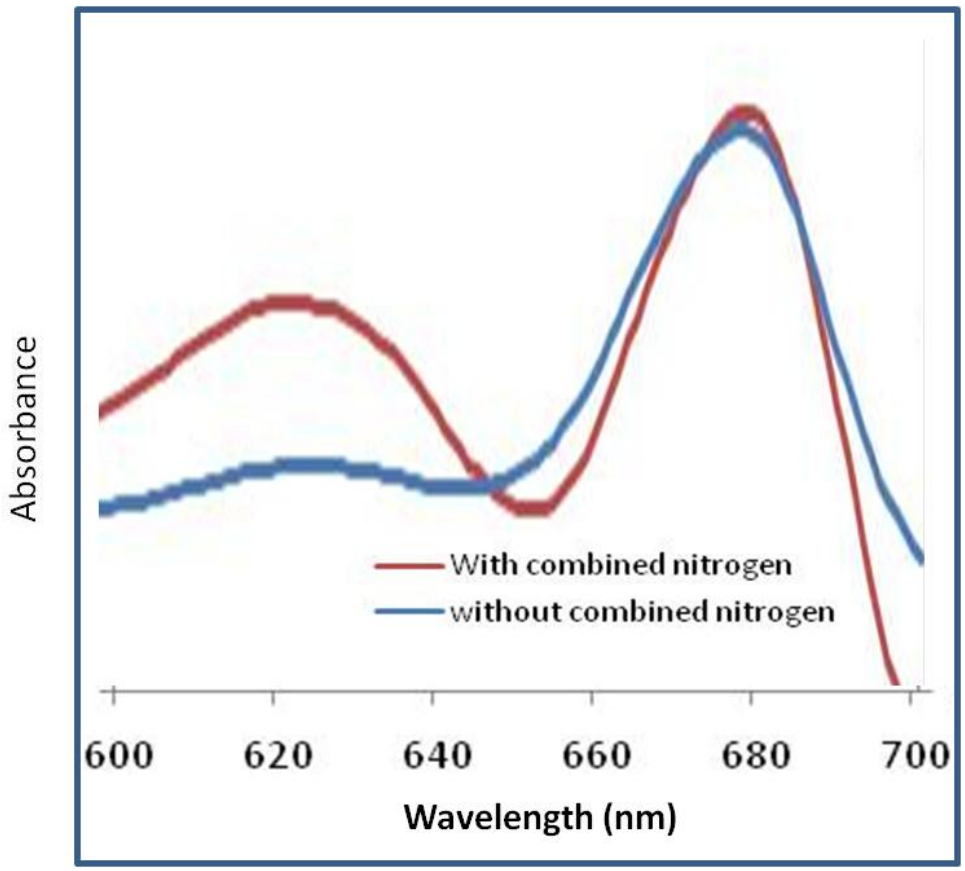

Fig. 3: Absorption spectra of *L. nodulosa* grown in MASM (red) and in MASM-0. The spectra were normalized to approximately the same peak height at 678 nm (chlorophyll a absorbent peak). Both cultures were grown in dim light (~15 µmol photons m^{-2} sec^{-1}).

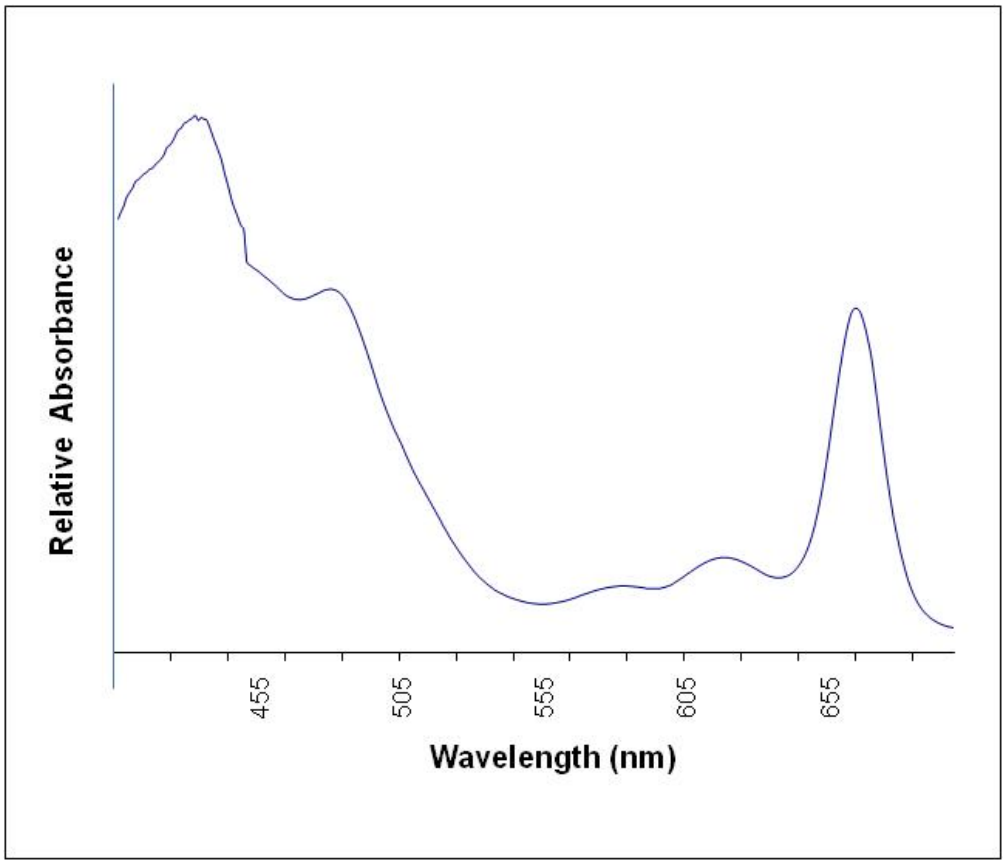

Fig. 4: Absorption spectrum of a lipid extract of *L. nodulosa* measured in 80% acetone.

B. Microscopy

1. Light and confocal microscopic observations

L. nodulosa is a filamentous cyanobacterium. Filaments are very long, typically consisting of 200 - 300 cells, and sometimes containing over 500 cells in undisturbed cultures (Fig. 5). Filaments are straight or gently curved, and occasionally are bent or folded. No false branching, heterocysts, akinetes or true

branching were seen in any filament, regardless of culturing conditions. Filaments always contained a well-defined sheath, easily observed by light microscopy in the presence of India ink and by DAPI/chlorophyll florescence microscopy (Figs. 6B, 6C and 7). Trichomes always occurred singly within a sheath. Some regions of the filaments had a much thicker sheath than other regions, which was especially evident in slow-growing cultures (Figs. 6C & 7).

Individual cells were barrel-shaped, with evident constriction of the trichome at cross-walls between cells (Figs. 6A & 6B). Trichomes were 1.1 - 1.5 µm in diameter and individual cells were 1.2 - 2.4 µm in length, depending on the extent of cell elongation since the last division (Fig. 6A). Under dim red light, cell division appeared to be arrested while growth continued, since cells became elongated (Fig. 6B).

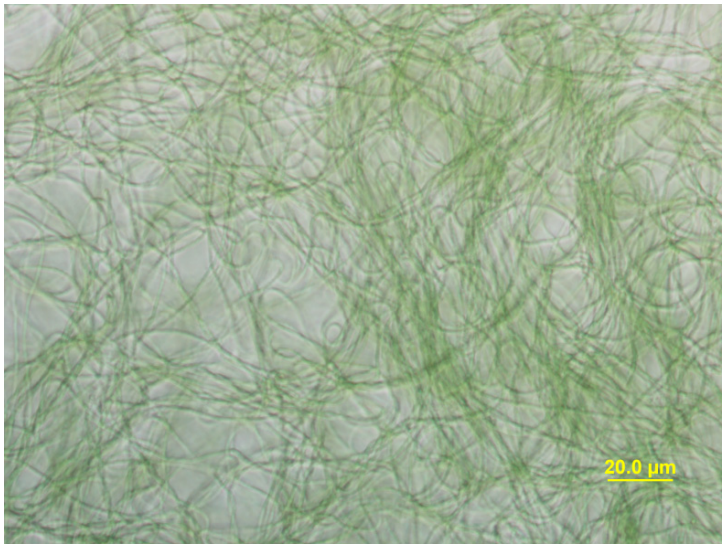

Fig. 5: Filaments of *L. nodulosa* that were cultured at high light intensity (60 µmol photons m^{-2} sec^{-1}) and observed by bright-field microscopy at 100X magnification. Note the lack of nodules.

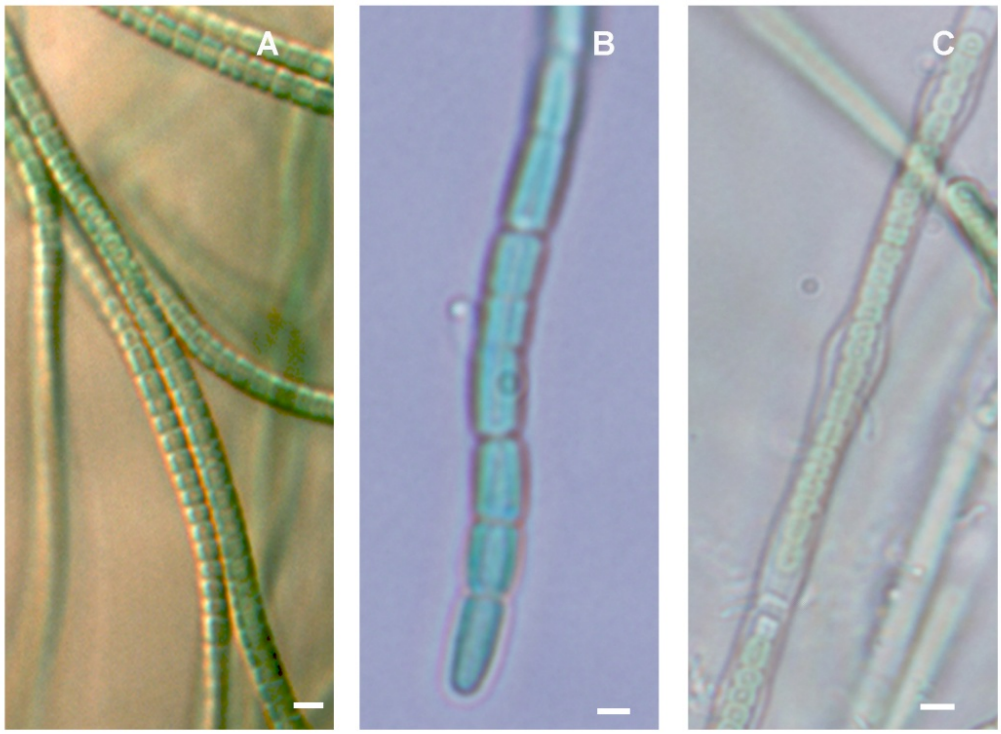

Fig. 6: Appearance of cells within filaments of *L. nodulosa*. A: DIC (Normaski) image (magnification 400X) of filaments in a culture grown at high light intensity. Note the nearly isodiametric disc-shaped cells with constrictions at cross-wall; B: Bright-field image (magnification 1,000X) of a typical filament in a culture grown in red light (cut-off λ = 575 nm) at low intensity (15 µmol photons m^{-2} sec^{-1}). Note the elongated shape and blue color of cells; C: Bright-field (high contrast) image (magnification 1,000X) of filaments in a culture grown at high light intensity. Note the variable thickness of the sheath that surrounds a trichome. Scale bars (A, B & C) = 2 µm. Note the midline devoid of chlorophyll running parallel to the length of trichomes in some profiles (seen in A, B, & C).

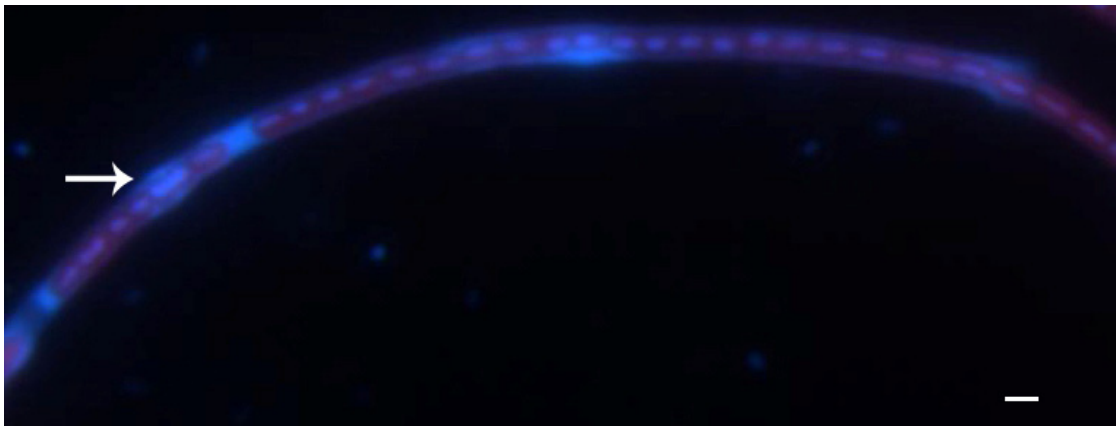

Fig. 7: A filament of *L. nodulosa* stained with DAPI and viewed by fluorescence microscopy at 400X magnification. Note the blue staining of thickened areas of the sheath (arrow) as well as the center nucleoid area of individual cells. Red color is auto-fluorescence of chlorophyll. Scale bar = 2 μm.

Cell division appeared to occur uniformly along entire filaments since incipient cross walls were seen in nearly all cells of a filament at the same observation times. Often two or more adjacent intercalary cells became necrotic, leaving an extended length of empty sheath. At other locations along the filament two non-adjacent necrotic sites isolated a single living cell or several adjacent cells. Necrotic sites appeared to represent positions of filament breakage, providing a mechanism for reproduction (Fig. 8). Short filaments consisting of less than 10 cells were always seen in actively-growing cultures. These hormogonia, which were likely derived from breakage of longer filaments, had no

detectable sheath and displayed a trembling motion. This motion was sensitive to temperature and stopped completely in cultures brought to less than 5°C.

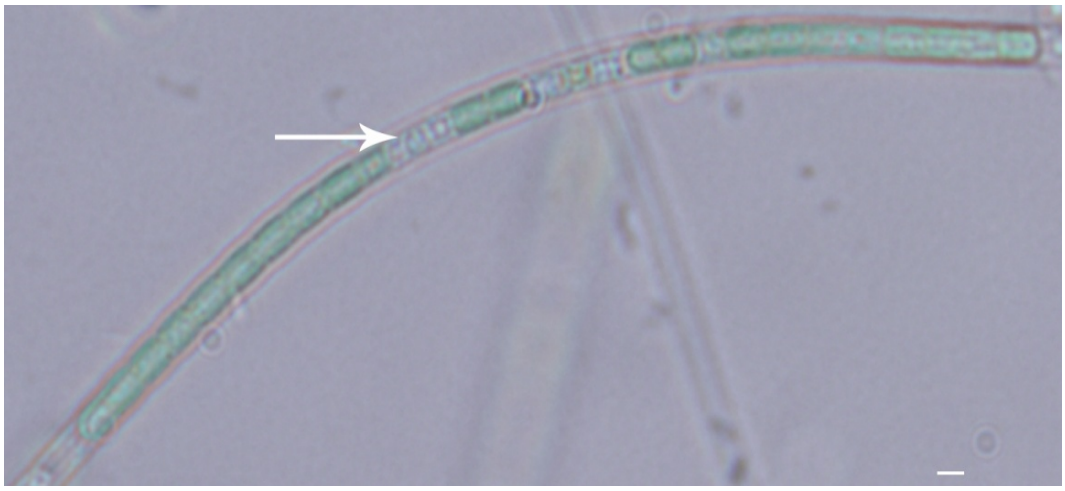

Fig. 8: Bright-field (high contrast) image (400X magnification) of a *L. nodulosa* filament. Note regions of necrotic cells (arrow). Scale bar = 1 μm.

In cultures growing at low light intensities (less than 15 μmol photons m^{-2} sec^{-1}), portions of filaments form discrete aggregates (nodules) of highly variable size and shape (Fig. 9).

Nodules sometimes exceed 15 μm in diameter. They consist of regions of a filament that are folded and twisted into globular structures (Fig. 10). They occur in both liquid suspension and on agar slants, but are much more pronounced in cultures grown on solid medium.

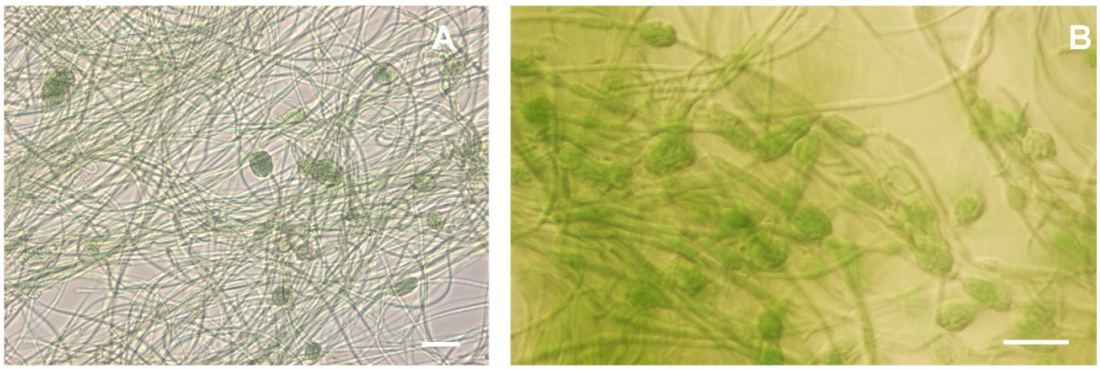

Fig. 9: Filaments of *L. nodulosa* that were cultured at low light intensity and observed by bright-field microscopy at 100X magnifications (Panel A) or at 400X magnification (Panel B). Note the well-defined tightly aggregated regions of filaments. Scale bars = 20 μm.

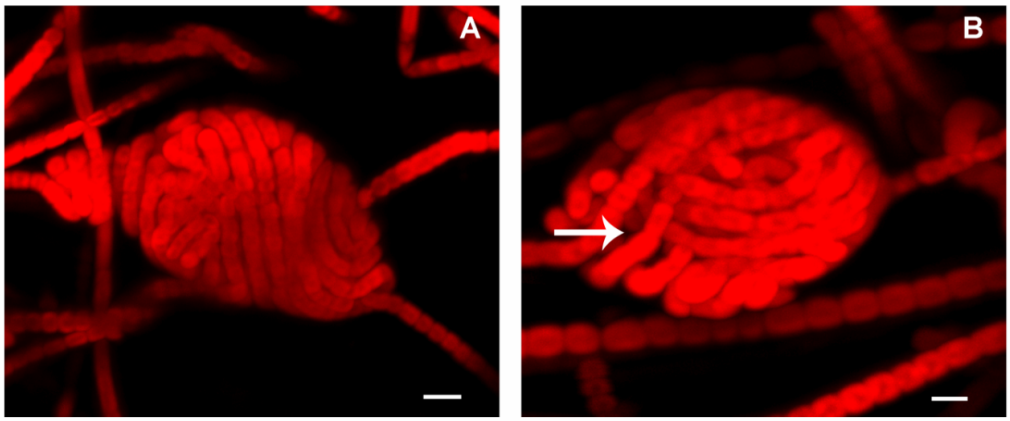

Fig. 10: 3-D confocal fluorescence images of nodules (magnification 600X). The red color is due to auto-fluorescence of chlorophyll. A: Note the highly irregular shape of the nodule and the parallel organization of regions of the filament within the nodule. B: Note the spaces between adjacent portions of the filament within the nodule that appear empty (arrow). Scale bars = 2 μm.

Nodules are quite stable and remain intact during preparations for light or electron microscopy without any special precautions. Large nodules could be disrupted by vigorous homogenization with a hand-held tissue grinder equipped with a Teflon pestle, but smaller aggregates remained stable even under this shear force. Except for the requirement of low light intensity, nodule formation did not seem to depend on culturing conditions. For example, neither culture temperature nor culture salinity had a noticeable effect on the formation of nodules. Nodules disappeared from cultures within 1 - 2 weeks after the cultures were transferred from low light intensity (6 μmol photons m^{-2} sec^{-1}) to high light intensity (60 μmol photons m^{-2} sec^{-1}), and no nodules were formed in cultures growing at the high light intensity. Nodules appeared 2 - 3 weeks after a culture growing at high light intensity was transferred to low light intensity.

2. Electron microscopic observations

Scanning electron microscopy showed that filaments have a sheath of variable thickness with a well-defined outer boundary, and with bacteria often adhering to the external surfaces (Fig. 11). Filaments also often appear twisted when viewed by scanning electron microscopy.

Samples prepared for transmission electron microscopy by glutaraldehyde fixation revealed structural details of the sheath surrounding trichomes (Fig. 13B). However, the intracellular ultrastructure was not seen distinctly by this

method. Samples prepared by freeze-fixation revealed individual thylakoids and other intracellular structural details (Fig. 12), but the surrounding sheath was not clearly resolved by this method.

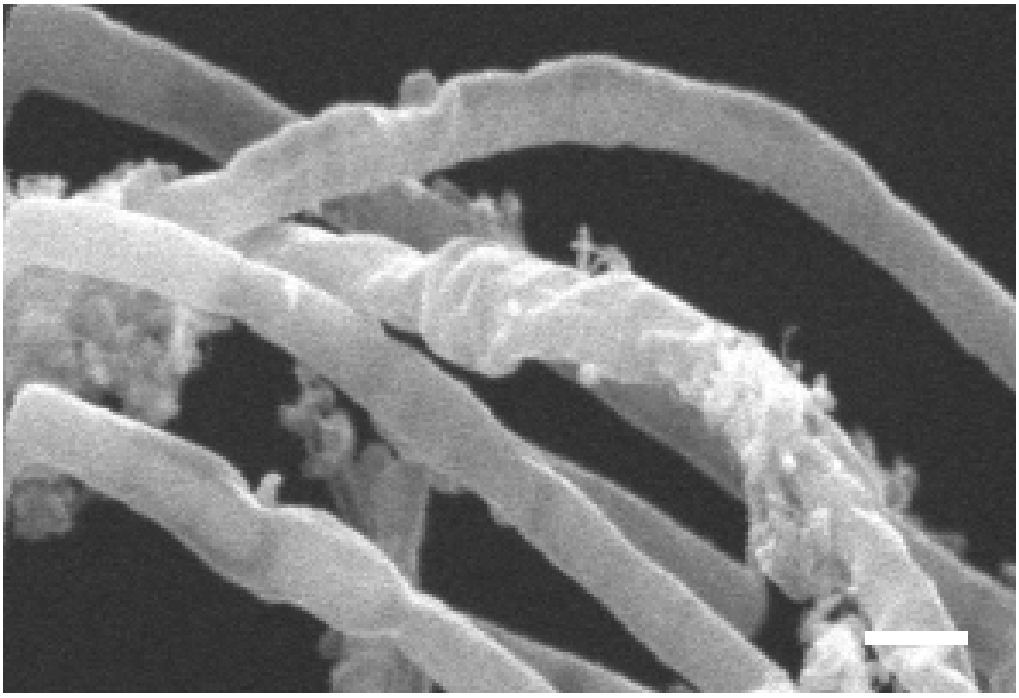

Fig. 11: Scanning electron micrograph showing filaments of *L. nodulosa* with sheaths of variable thickness and bacteria adhering to external surfaces. Scale bar = 2 μm.

Transmission electron micrograph of *L. nodulosa* demonstrates a typical cyanobacterial ultrastructure, with a central nucleoid and inclusions of cyanophycin bodies. Gas vesicles were not observed by transmission electron microscopy or any other microscopic technique. Thylakoids occur only at the

periphery of cytoplasm (Fig. 12). The thylakoids remained well separated along the lateral portions of cells, but appeared to merge at one or both ends. The distribution and arrangement of thylakoids appeared the same in cells of non-aggregated portions of trichomes and in the cells within nodules (Figs. 12 & 14).

Filaments of *L. nodulosa* seen in cross-section by transmission electron microscopy often appeared flattened, with a flanged sheath on two opposite sides (Fig. 12). Sections of *L. nodulosa* cells examined by transmission electron microscopy revealed a typical laminated cyanobacterial wall structure. A prominent sheath always surrounded cells, but in most sections appeared separated from the wall by an extensive electron-transparent layer (Fig.13A). Although the composition of the sheath was somewhat variable, it typically consisted of an inner diffuse layer of variable thickness, surrounded by an electron dense area approximately 20 nm thick. A fibrous layer surrounded the electron dense layer, which contained individual helical fibrils. The thickness and density of the fibrous layer were variable (Fig. 13B).

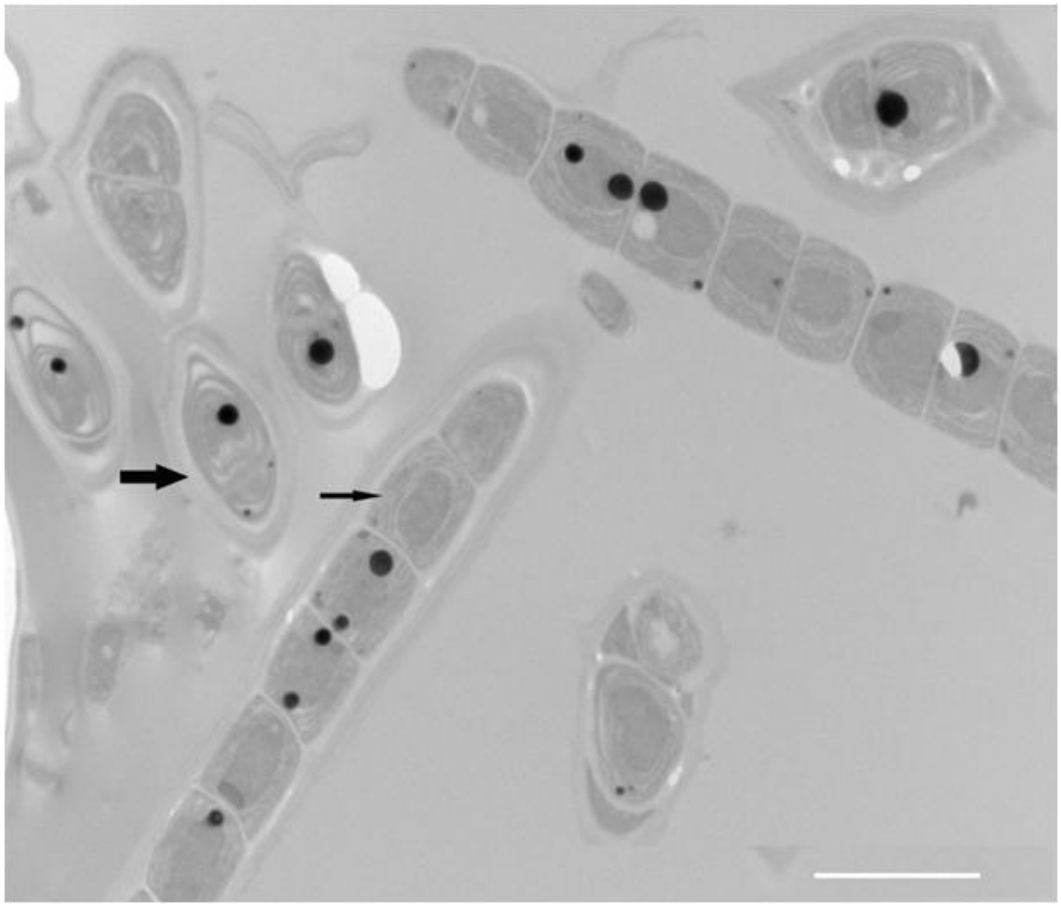

Fig. 12: Transmission electron micrograph of sectioned filaments. Note the flattened appearance of filaments seen in cross section (thick arrow). Also note that thylakoids appear to merge at one or both ends of cells (thin arrow). Dark staining spherical bodies are cyanophycin. Light-colored bodies are polyphosphate, bright areas of irregular shape are holes in the section prepared for transmission electron microscopy. Scale bar = 2 μm

The folded and twisted portion of the filament within a nodule retains its sheath and additional sheath material surrounds the entire nodule (Fig. 14). The ultrastructure of the external portion of the sheath surrounding the entire nodule appears identical in cross section to the sheath surrounding an individual trichome. Often areas of low electron density occur within nodules, external to protoplasts but interior to the dense sheath that surrounds the entire nodule.

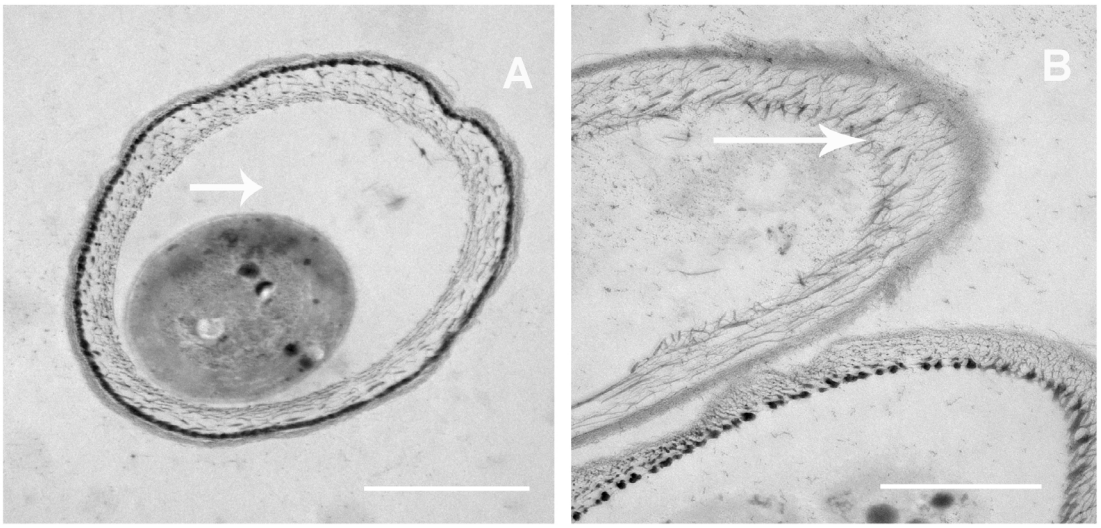

Fig. 13: Transmission electron micrographs of *L. nodulosa* cells, showing the ultrastructure of sheaths. Note the electron-transparent region between the protoplast and the sheath. Also note the laminated structure of the sheath. Scale bars = 1 μm. A: Cross section. B: Tangential section. Note the helical fibrils in the outer region of the sheath.

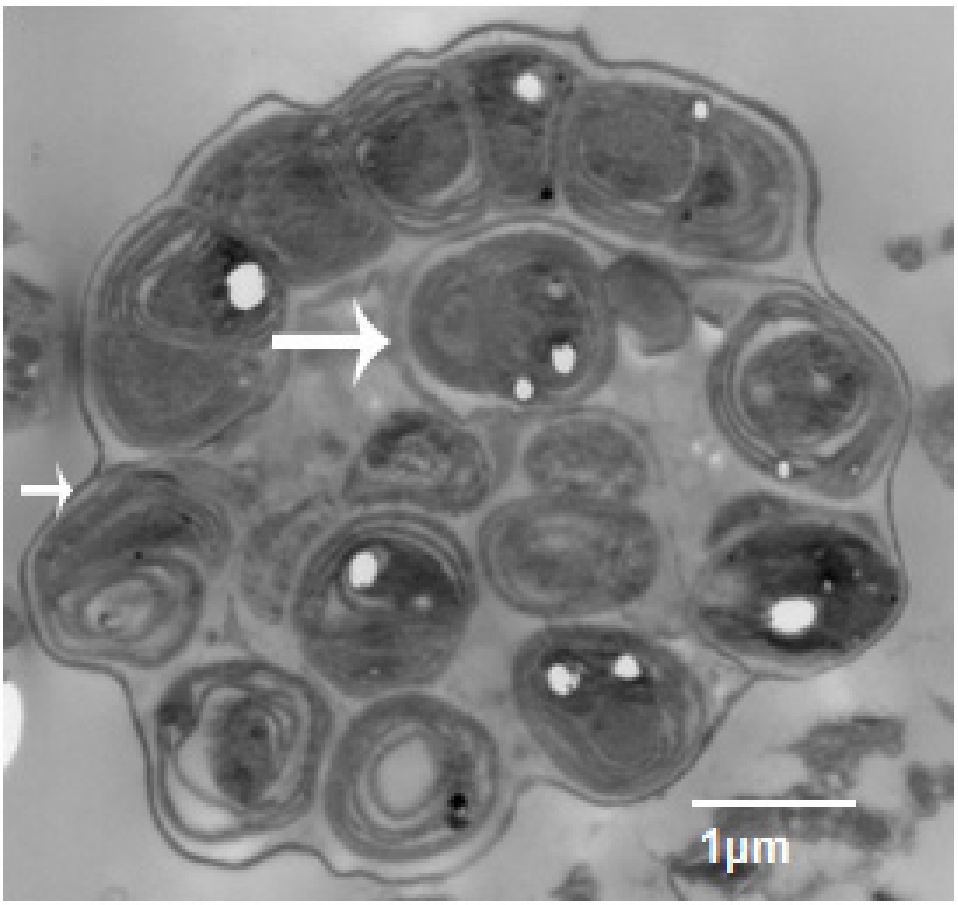

Fig. 14: Transmission electron micrograph of cross section of a nodule. Note the sheath around each individual portion of the filament (large arrow) and the common sheath surrounding the entire nodule (small arrow). Scale bars = 1 µm.

The sheath that surrounded each nodule has a distinct and well-defined boundary (Fig. 15).

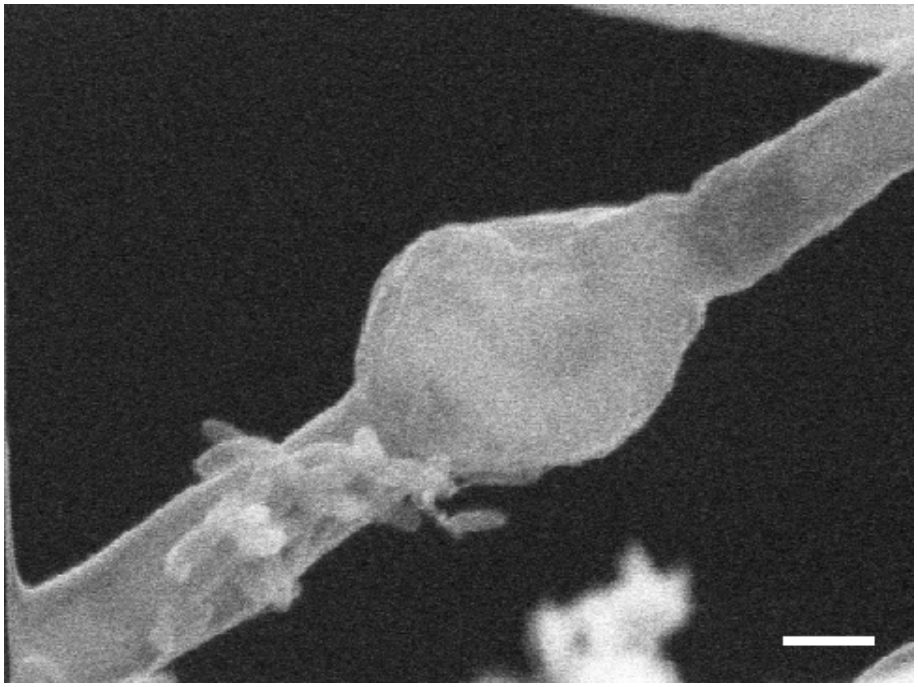

Fig.15: Scanning electron micrograph of a nodule. Note the distinct common sheath with a well-defined boundary surrounding the entire nodule. Bacteria are seen adhering to the external surface of a region of the filament adjacent to the nodule. Scale bar = 1 μm.

C. Molecular phylogenetic analyses

The 16s rDNA and ITS sequences of *L. nodulosa* were determined in order to infer relationships of *L. nodulosa* to other cyanobacteria. Standard procedures were used to extract DNA, amplify specific regions of the DNA, and determine their sequences (Materials and Methods). The 16S rDNA and ITS sequences of *L. nodulosa* are listed in Appendix III.

A search of NCBI GenBank (BLAST search) was conducted (October 14th, 2007) to identify homologous 16s rDNA sequences, using the "highly similar sequences" option. The search results showed that the sequences most similar to 16s rDNA of *L. nodulosa* were from strains previously identified as *Leptolyngbya*. All *Leptolyngbya* 16s rDNA sequences for which the BLAST results showed ≥ 90% sequence homology, and all other sequences showing ≥ 91% homology, were used to construct phylogenetic trees. Fig. 16 shows a phylogenetic analysis of the data using N-J analysis. A virtually identical tree was generated using Maximum Likelihood (Aldrich 1997) analysis (not shown). In view of the very limited number of morphological characters that are available to distinguish genera of cyanobacteria, it may not be surprising that some strains of non-heterocystous filamentous cyanobacteria previously identified as *Oscillatoria, Phormidium* and *Plectonema* also fall within the *Leptolyngbya* clade. The *Oscillatoria neglecta* shown to be very closely related to *L. nodulosa* according to its 16s rDNA sequence (Fig. 16) has been re-named as *Jaaginema*

because of its very thin trichome diameter (Anagnostidis & Komárek 1988). Thus, except for its reported lack of an external sheath, it is indistinguishable from *Leptolyngbya*.

Fig. 16 demonstrates that strains identified as *Leptolyngbya* fall into at least 2 major clades. The type species, *L. boryana*, falls into one clade while *L. nodulosa* falls into the other. Thus, it may be appropriate to divide the genus *Leptolyngbya* into 2 separate genera when sufficient phylogenetic information becomes available.

A phylogenetic tree showing sequences most homologous to the *L. nodulosa* ITS sequence is presented in Fig. 17. At the time of this analysis (October 14th, 2007), few ITS sequences similar to that of *L. nodulosa* had been published in GenBank. Thus, although confirming that *Leptolyngbya* falls into the LPP group of non-heterocystous cyanobacteria, little detailed systematic information is revealed. The ITS sequence of *L. nodulosa* has two inserts, a tDNA^{Ala} and a tDNA^{Ile} sequence (Appendix III). The ITS of many cyanobacteria contain 1 - 3 embedded tDNA sequences, although an insufficient number of different species have been identified to determine the phylogenetic significance of these insertions.

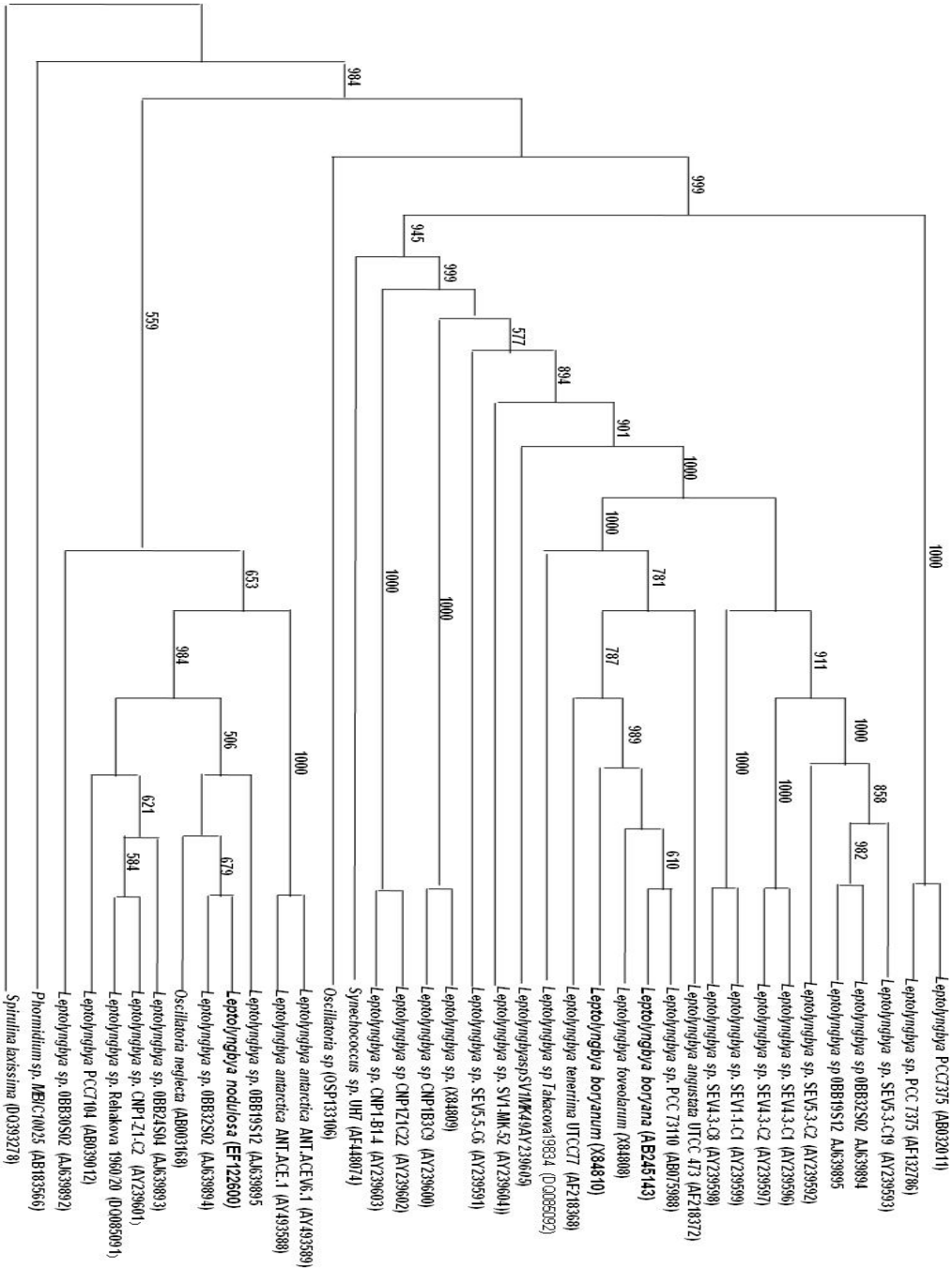
Leptolyngbya PCC7375 (AB032011)
Leptolyngbya sp. PCC 7375 (AF132786)
Leptolyngbya sp. SEV5-3-C19 (AY239593)
Leptolyngbya sp 0BB32502 AJ639894
Leptolyngbya sp 0BB19512 AJ639895
Leptolyngbya sp. SEV5-3-C2 (AY239592)
Leptolyngbya sp. SEV4-3-C1 (AY239596)
Leptolyngbya sp. SEV4-3-C2 (AY239597)
Leptolyngbya sp. SEV1-1-C1 (AY239599)
Leptolyngbya sp. SEV4-3-C8 (AY239598)
Leptolyngbya angustata UTCC 473 (AF218372)
Leptolyngbya sp. PCC 73110 (AB075988)
Leptolyngbya boryana (AB245143)
Leptolyngbya foveolarum (X84808)
Leptolyngbya boryanum (X84810)
Leptolyngbya tenerima UTCC77 (AF218368)
Leptolyngbya sp Takacova19834 (DQ085092)
Leptolyngbyasp SVMK49AY239605)
Leptolyngbya sp. SV1-MK-52 (AY239604)
Leptolyngbya sp. SEV5-5-C6 (AY239591)
Leptolyngbya sp. (X84809)
Leptolyngbya sp CNP1B3C9 (AY239600)
Leptolyngbya sp CNP1Z1C22 (AY239602)
Leptolyngbya sp. CNP1-B1-4 (AY239603)
Synechococcus sp. UH7 (AF448074)
Oscillatoria sp (OSP133106)
Leptolyngbya antarctica ANT.ACEV6.1 (AY493589)
Leptolyngbya antarctica ANT.ACE.1 (AY493588)
Leptolyngbya sp. 0BB19512 (AJ639895
Leptolyngbya nodulosa (EF122600)
Leptolyngbya sp. 0BB32502 (AJ639894)
Oscillatoria neglecta (AB003168)
Leptolyngbya sp. 0BB24504 (AJ639893)
Leptolyngbya sp. CNP1-Z1-C2 (AY239601)
Leptolyngbya sp. Rehakova 1960/20 (DQ085091)
Leptolyngbya PCC7104 (AB039012)
Leptolyngbya sp. 0BB30502 (AJ639892)
Phormidium sp. MBIC10025 (AB183566)
Spirulina laxissima (D0393278)
1000
999
984
945
999
577
894
901
1000
1000
781
787
989
610
911
1000
1000
1000
858
982
1000
1000
653
984
506
679
584
621
559
1000
1000
1000
1000

Fig. 16: Neighbor-Joining phylogenetic tree based on 16s rDNA sequences, showing relationships of *L. nodulosa* with other cyanobacteria. *Sprirulina laxissima* was used as outgroup. Numbers above the branches are bootstrap support values (1,000 data sets); values less than 500 are not shown. *L. nodulosa* and two strains of type strain, *L. boryana* (one identified as *L. boryanum*), are shown in bold.

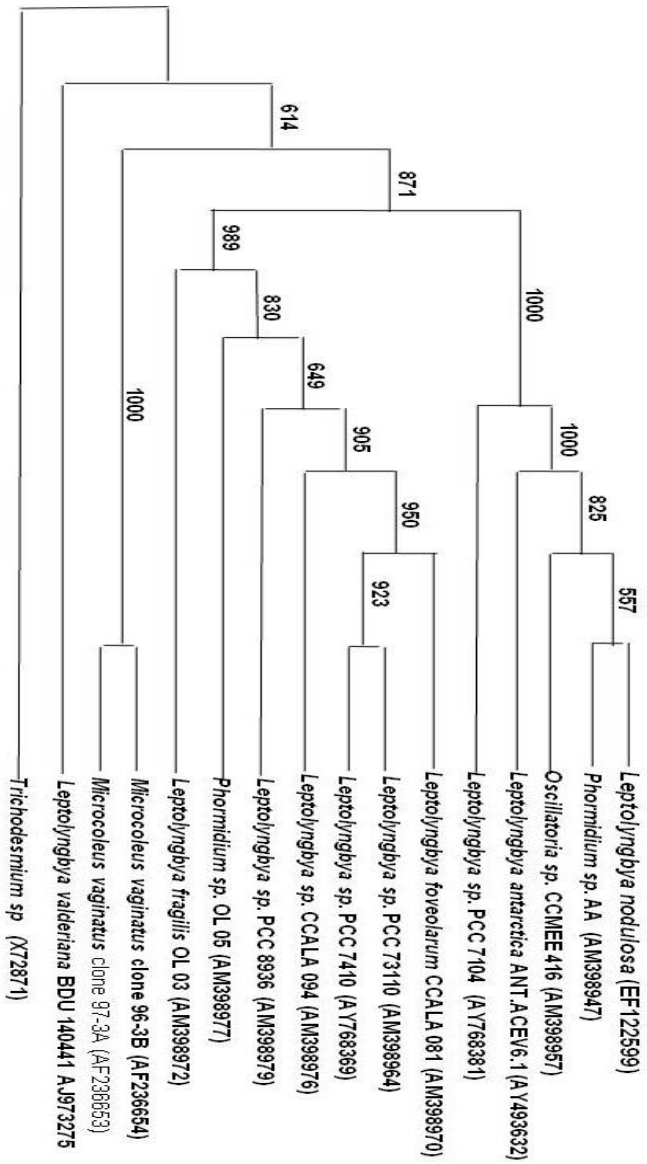

Fig. 17: Neighbor-Joining phylogenetic tree based on ITS sequences, showing relationships of *L. nodulosa* with other cyanobacteria. *Trichodesmium sp.* was used as outgroup. Numbers above the branches are bootstrap support values (1,000 data sets).

D. Assignment of the strain

Comparisons of 16s rDNA, the 16s – 23s internal transcribed spacer (ITS) sequence, and morphological characteristics of this strain to corresponding features of other cyanobacteria indicate that it is a member of the genus *Leptolyngbya*. Differences in DNA sequences and the occurrence of nodules indicate that it is not closely related to the type species, *L. boryana*, and should be assigned a distinct specific epithet. Accordingly a new name, *Leptolyngbya nodulosa*, has been assigned to this organism (Li & Brand 2007).

Leptolyngbya nodulosa Li and Brand, *sp. nov.*
DIAGNOSIS: Proprietatibus descriptis pro genere sed differt characteribus sequentibus: filamentis distincte complanatis e cellulis aliquot centum cylindricis 1.2 – 2.4 μm longis 1.1 – 1.5 μm diametro compositis, plicatis in fabricas compactas per vaginam distinctam omnino circumcinctas; reproductione per fractione trichomatum ad situs cellularum necroticarum; et hormogonia motibus tremulis.

With features described for the genus but different in the following characters: in its filaments distinctly flattened, composed of several hundred cylindrical cells 1.2 - 2.4 μm long by 1.1 - 1.5 μm diameter; in the occurrence along filaments of discrete compact structures of variable shape and size that consist of a portion of

the filament folded into a tight aggregate and entirely surrounded by a distinct sheath; in its reproduction by fragmentation of trichomes at sites of necrotic cells; and in its hormogonia with trembling movements.

Iconotype: Figs. 1 – 10 (Li & Brand 2007).

Holotype isolated from the South China Sea (coordinates 114° 16’ E, 22° 70’ N) at a depth of approximately 10 m. Deposition: cryopreserved living culture in the Culture Collection of Algae at the University of Texas at Austin (Accession number UTEX 2910).

Etymology: Specific epithet is the Greek name for “little node” or “nodule”, a characterization of the distinguishing feature of this organism.

E. Dinitrogen fixation

1. Growth of *L. nodulosa* in medium devoid of combined nitrogen

Non-axenic cultures of *L. nodulosa* could be maintained indefinitely (for at least two years) at low light intensity in medium devoid of any combined nitrogen source. Cultures grew slowly (estimated doubling time 3 weeks) at a low intensity (13 - 15 μmol photons m^{-2} sec^{-1}), but did not survive incubation at high light intensity (60 μmol photons m^{-2} sec^{-1}). Cultures grown in the presence of either NH_4^+ or NO_3^- appeared deep green in color while cultures exposed to N_2 as the only nitrogen source were devoid of detectable phycobillin proteins (Fig. 3) and appeared yellow green in color (Fig. 18). Axenic cultures of *L. nodulosa* did not grow in medium devoid of a combined nitrogen source and died within one month, even at low light intensities. Thus, cultures of *L. nodulosa* are apparently able to fix dinitrogen, but only at low light intensities and only in cultures that also contain heterotrophic bacteria.

2. Diurnal cycling of dinitrogen fixation

Measurement of acetylene reduction is a reliable method for determining rates of dinitrogen fixation in organisms that contain dinitrogenase (Mague 1978). This method takes advantage of the observation that acetylene complements dinitrogen as a substrate in the dinitrogenase reaction.

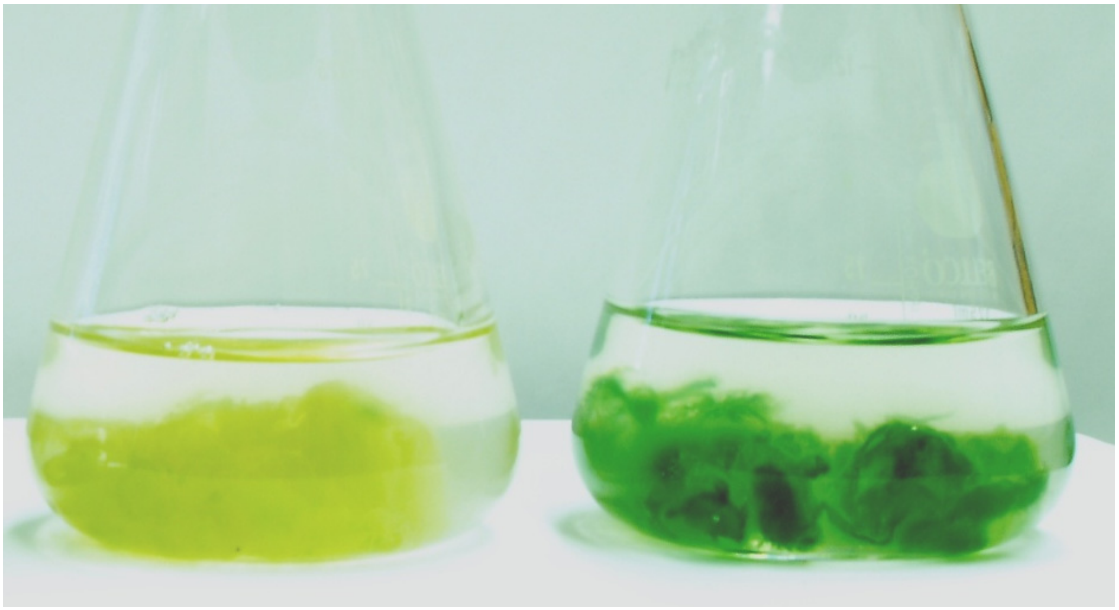

Fig. 18: Cultures of *L. nodulosa* grown in MASM-0 (left) and MASM (right). Both cultures were maintained at 25°C without disturbance for several months under a diurnal light cycle (12 h light:12 h dark) at light intensity of ~13 μmol photons m^{-2} sec^{-1}.

In the presence of both N_2 and C_2H_4, these two substrates function together to produce ammonium and ethylene as final products in the approximate ratio of $3NH_4^+$:$1C_2H_4$ (Seitzinger & Garber 1987). Ethylene is a gas that is released from cells, while ammonium generally remains within cells where it is metabolized. Acetylene reduction assays are performed under the same conditions that facilitate dinitrogen fixation in the absence of acetylene, and the rate of ethylene production is measured by gas chromatography (Karl *et al*. 2002).

Non-axenic cultures of *L. nodulosa* contain an active dinitrogenase, as shown in Fig. 19. For this and other experiments designed to measure dinitrogenase activity, cultures were grown in nitrogen deficient medium (MASM-0) for at least one month prior to experiments (Appendix I). Cultures exposed to an illumination cycle of 12 h light:12 h dark expressed dinitrogenase activity diurnally, with most acetylene reduction activity in darkness. Activity was detected within 3 hours after an inactive illuminated sample was placed in darkness, and continued to rise for at least 9 hours of continuous darkness. Dinitrogenase activity began to decline immediately after illumination and was not detectable after 6 hours of illumination. Activity during a dark period was dependent on the light intensity during the preceding illumination period, with highest rates obtained after relatively low-intensity illumination (Fig. 19).

The absolute rates of ethylene production under these conditions, expressed per unit chlorophyll or per unit wet weight, were low, less than 5% of typical rates measured in heterocystous cyanobacteria such as *Anabaena* (Fig. 20). Data shown in Figs. 19 and 20 were for experiments conducted in the absence of bicarbonate. Rates of acetylene reduction in the presence of bicarbonate were approximately twice these rates (data not shown), but the pattern of acetylene reduction was the same. Because of these low rates, measured values of ethylene production varied greatly among replicate samples. Subsequent acetylene reduction assays were performed in the presence of bicarbonate.

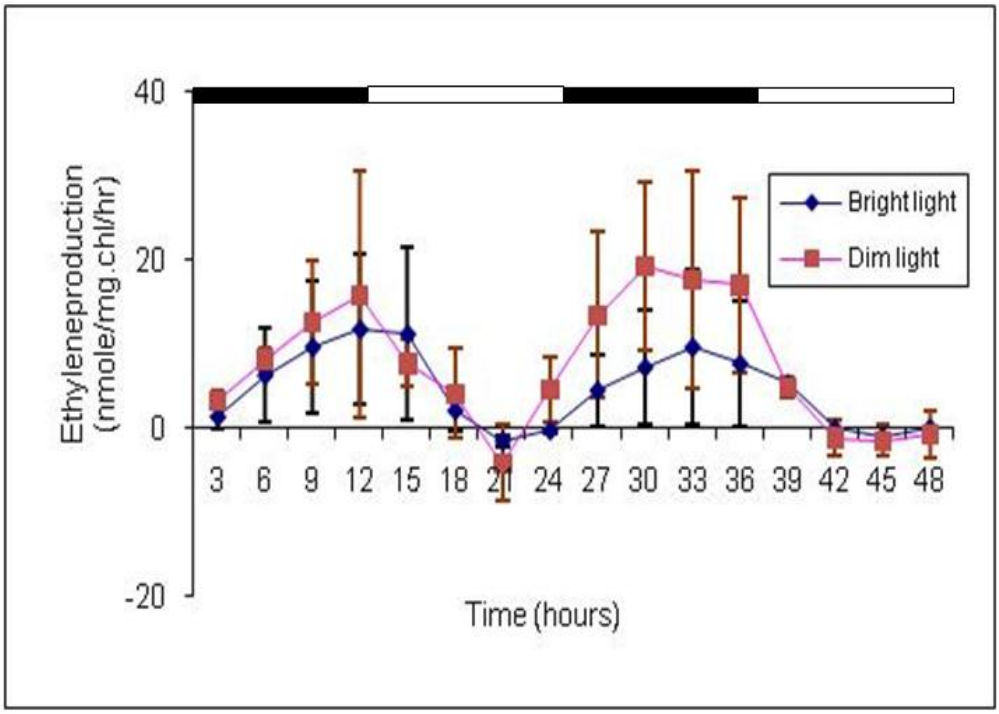

Fig. 19: Diurnal pattern of dinitrogen fixation in *L. nodulosa* cultures. The black and white bars represent dark and light periods, respectively. Light intensity of dim light was ~13 µmol photons m^{-2} sec^{-1} and of bright light was ~60 µmol photons m^{-2} sec^{-1}. Each point represents an average of the values measured for two cultures prepared in parallel.

The presence of ammonia (2 μM) completely suppressed dinitrogen fixation (Fig. 20). Dinitrogenase activity is not sustained indefinitely in continuous darkness. Maximum activity occurs 9 - 15 hours into the dark period, and then gradually declines to zero. Fig. 21 shows the continuous decline in dinitrogenase activity during a long period of darkness following a 12-hour light period. The first datum point of Fig. 21 (shown here as hour 27) represents dinitrogenase activity 15 hours into a dark period following 12 hours of light. Figs. 19 and 21 together demonstrate that both light and dark periods are required to establish conditions that permit sustained dinitrogen fixation.

The graphs shown in Fig. 19 represent assays performed in cultures that were maintained anaerobically. The pattern of dinitrogenase activity was unaltered, but the absolute amount of ethylene produced during a 24-hour diurnal illumination cycle was diminished, as the concentration of oxygen in the culture was increased (Fig. 22). Thus, dinitrogenase functions in an anaerobic and a micro-aerobic environment in *L. nodulosa* cultures, but not under aerobic conditions (21% O_2).

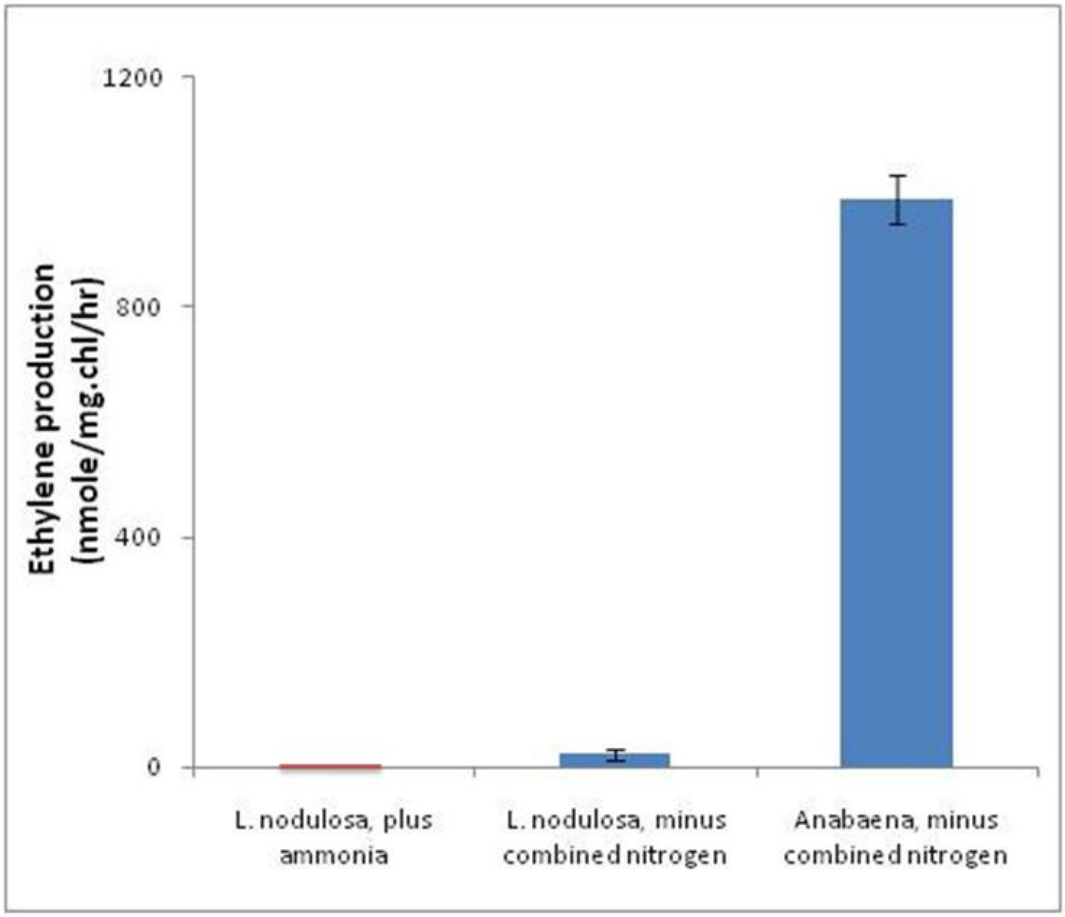

Fig. 20: Comparison of the rate of dinitrogen fixation in *L. nodulosa* cultures with rates in *Anabaena* PCC7120. Data are selected from the maximum individual average rate of each experiment. Error bars represent an average of two cultures prepared in parallel.

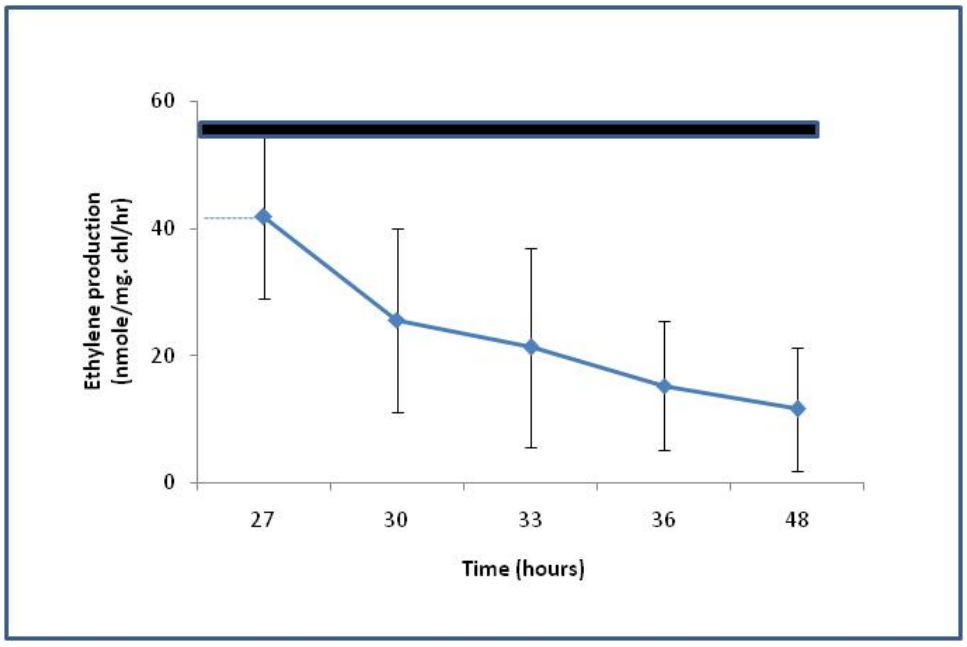

Fig. 21: Duration of dinitrogen fixation in continuing darkness subsequent to 12 hours of light (60 μmol photons m^{-2} sec^{-1}) followed by continuous darkness.

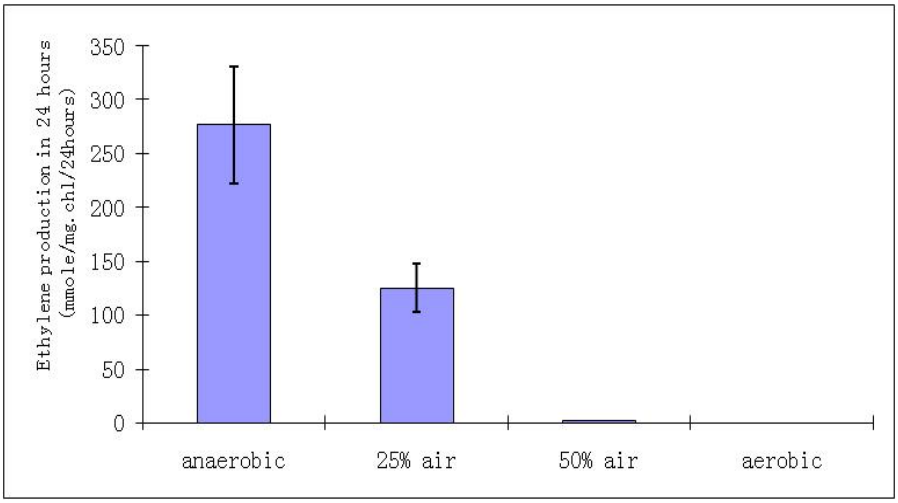

Fig. 22: Total amount of ethylene production during a 24-hour period of a diurnal illumination in cultures incubated under various concentrations of atmospheric O_2. Each bar represents an average of two experiments, with error bars representing the variation of the duplicate experiments.

3. Oxygen uptake or evolution in cultures of *L. nodulosa*

The diurnal pattern (Fig. 19) and oxygen sensitivity (Fig. 22) of dinitrogenase activity in cultures of *L. nodulosa* suggest that photosynthetically produced oxygen may inactivate dinitrogenase. The rate of oxygen uptake or evolution as a function of light intensity in a culture grown in the absence of any combined nitrogen source is shown in Fig.23. The light compensation point occurs at an intensity of approximately 40 μmol photons m^{-2} sec^{-1}, with net oxygen evolution at higher intensities and net uptake at lower intensities. Therefore, it appears that net oxygen uptake occurs in cultures measured at low light intensity (*e.g.* 15 μmol photons m^{-2} sec^{-1}) and a net oxygen evolution occurs in cultures measured at high light intensity (*e.g.* 60 μmol photons m^{-2} sec^{-1}). The rate of net oxygen uptake at very low intensities or in darkness was remarkably high, suggesting an unusually active respiratory system in this organism (Fig. 23, Myers 1986). Since dinitrogenase is inactivated by O_2, these observations may explain why cultures cannot survive in a nitrogen-deficient medium at high light intensity, but tolerate some atmospheric oxygen in darkness (Fig. 24), since they consume oxygen.

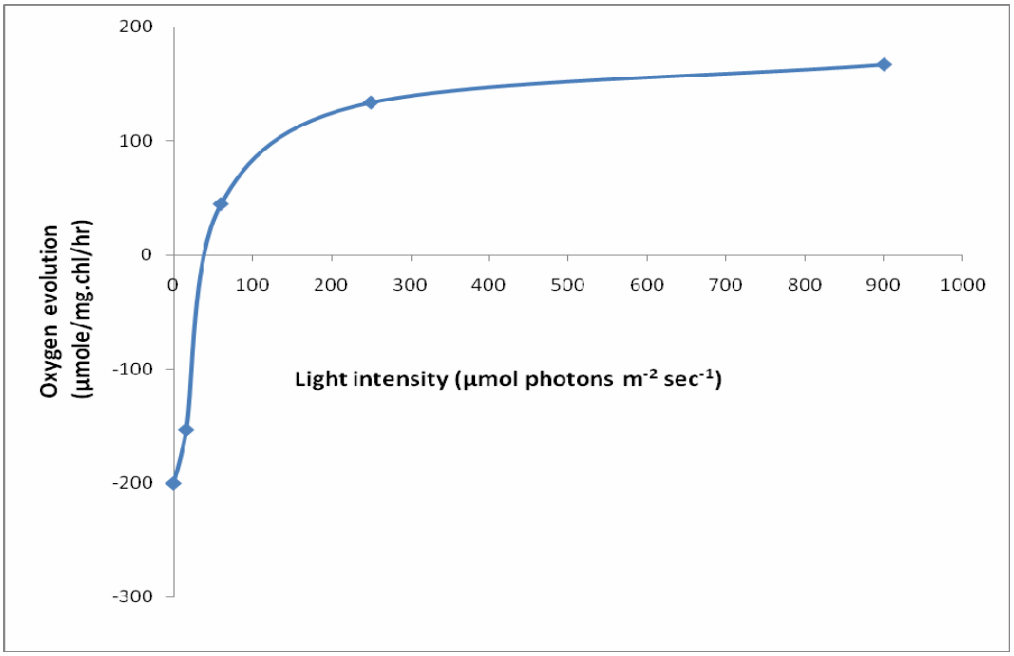

Fig. 23: Rate of oxygen evolution as a function of light intensity in a culture of *L. nodulosa* grown in MASM-0 medium.

4. The effect of oxygen concentration and DCMU on rates of dinitrogen fixation

Oxygen not only affects the total amount of dinitrogen fixed in a 24-hour light-dark cycle (Fig. 22), but also the maximum rate of dinitrogen fixation (Fig. 24). Substantial rates of ethylene production were seen under anaerobic or micro-aerobic (25% of atmospheric oxygen), but almost no ethylene was produced in the presence of 50% of atmospheric oxygen. Oxygen electrode assays demonstrated that DCMU, at a concentration of 50 µM, completely inhibits photosynthesis (data not shown). Cultures incubated in 50 µM DCMU over a 24-hour diurnal illumination cycle had maximum rates of dinitrogen fixation

~9 times as high as cultures incubated under identical conditions in the absence of DCMU (compare “anaerobic” and “DCMU” bars in Fig. 24). The total amount of acetylene reduced during a 24-hour diurnal illumination cycle in the presence of DCMU was typically ~10 times more than the amount reduced when no DCMU was present.

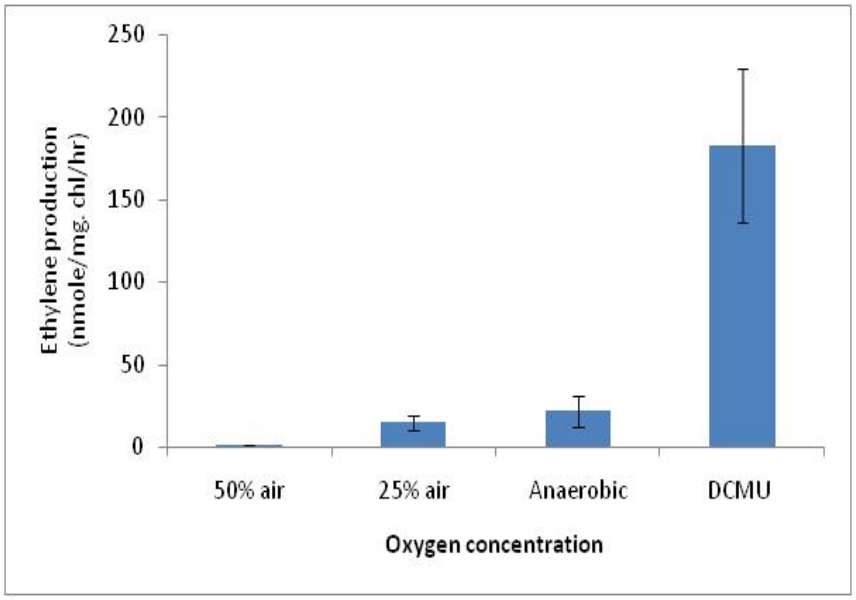

Fig. 24: Comparison of maximum rates of dinitrogen fixation by *L. nodulosa* cultures under various concentrations of oxygen, or in the presence of DCMU under anaerobic conditions. All assays were in cultures incubated at a light intensity of 60 µmol photons m^{-2} sec^{-1}. Each bar represents an average measurement of two cultures prepared in parallel.

5. Diurnal cycle of dinitrogen fixation in the presence of DCMU

The herbicide diuron (DCMU) is a potent inhibitor of oxygenic photosynthesis which selectively blocks photosynthetic electron transport at the reducing side of Photosystem II (Van Reensen 1982). Surprisingly, most dinitrogen fixation occurred in the light in the presence of DCMU (Fig. 25). Dinitrogen fixation rates increased rapidly to a high value in a light period following a dark period, then quickly returned to low level in the following dark period. The light-dependent rate of dinitrogen fixation in the presence of DCMU was greater than one-fifth of the value obtained in a heterocystous cyanobacterium in the absence of DCMU (Figs. 20 & 24). The inset of Fig. 25 shows that the rate dinitrogen fixation during darkness in the presence of DCMU is very similar to the rate in the absence of DCMU.

In short term measurements, DCMU, at 25 μM, was shown to inhibit approximately 30% of *L. nodulosa* oxygen evolution. That concentration of DCMU did not kill cultures grown under an illumination cycle of 12 h light:12 h dark at light intensity of 60 μmol photons m^{-2} sec^{-1} (data not shown), but did cause a complete reversal of the day-night dinitrogenase activity, as compared to activity in the absence of any DCMU. It also greatly enhanced the maximum rate of ethylene production (Fig. 26). At 100 μM, DCMU completely inhibits oxygen evolution in short term experiments; it retards growth but does not kill cultures in long term experiments. This concentration of DCMU has a similar effect as does 25 μM, although dinitrogenase activity is somewhat lower during the first of two

photoperiods (Fig. 26). DCMU at 400 μM completely kills cultures within 2 weeks. At that concentration, dinitrogenase activity is almost, but not completely inhibited.

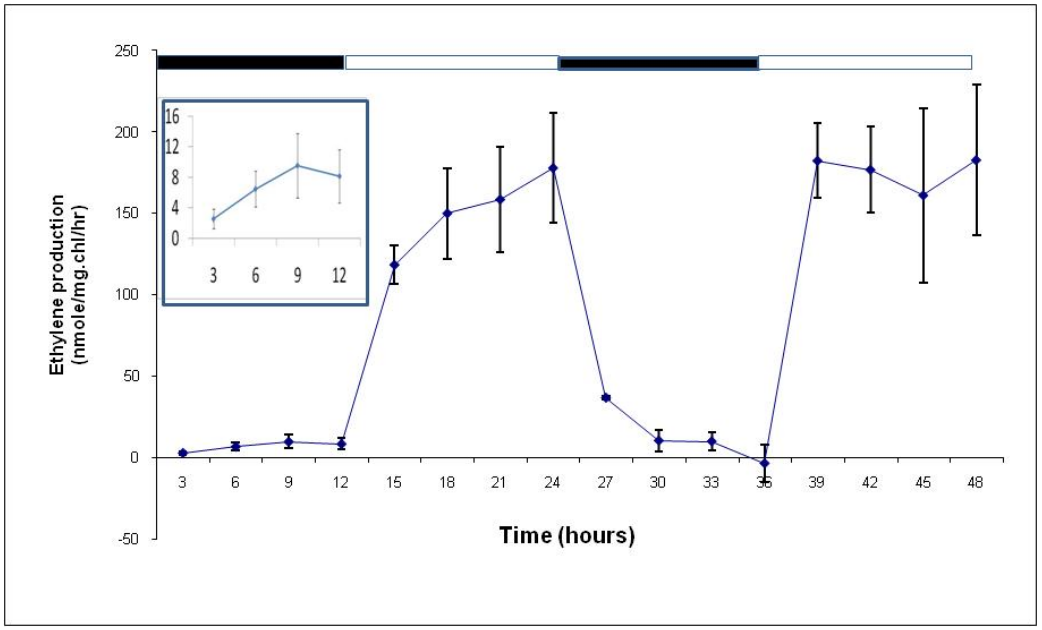

Fig. 25: Diurnal cycle of dinitrogen fixation in the presence of DCMU (100 μM). Light intensity during illumination was 60 μmol photons m^{-2} sec^{-1}. Each bar represents an average measurement of two cultures prepared in parallel.

6. Effects of chloramphenicol on dinitrogenase synthesis and activity

Chloramphenicol is a prokaryotic protein synthesis inhibitor known to block translation in cyanobacteria (Scherer *et al*. 1980). When chloramphenicol (20 μg/mL) was added to a culture of *L. nodulosa* in the light period of a diurnal cycle,

one hour before a dark period, dinitrogen fixation was suppressed during the following dark period. Fig. 27 demonstrates normal dinitrogenase activity during the dark period of a diurnal cycle prior to the addition of chloramphenicol, but nearly complete inhibition of activity during the next dark period after the addition of chloramphenicol. Chloramphenicol was added to the culture in ethanol, giving a final ethanol concentration of 0.15% (v/v) in the flask. A control experiment utilizing the same concentration of ethanol without chloramphenicol gave a pattern of dark-dependent dinitrogen fixation virtually identical to the pattern shown from hours 15 through 24 in Fig. 27 (data not shown).

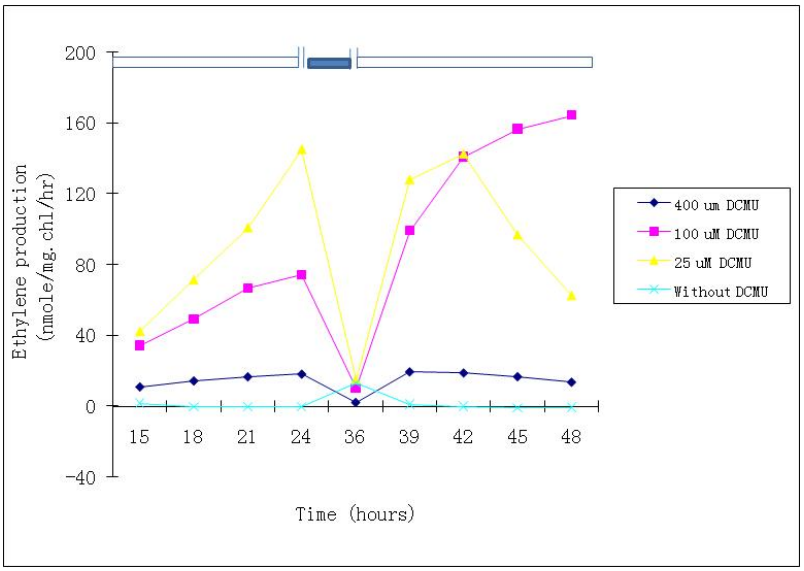

Fig. 26: Effects of several concentrations of DCMU on the diurnal cycle of dinitrogenase activity in *L. nodulosa*. Cultures were illuminated at 60 μmol photons m^{-2} sec^{-1} during light periods.

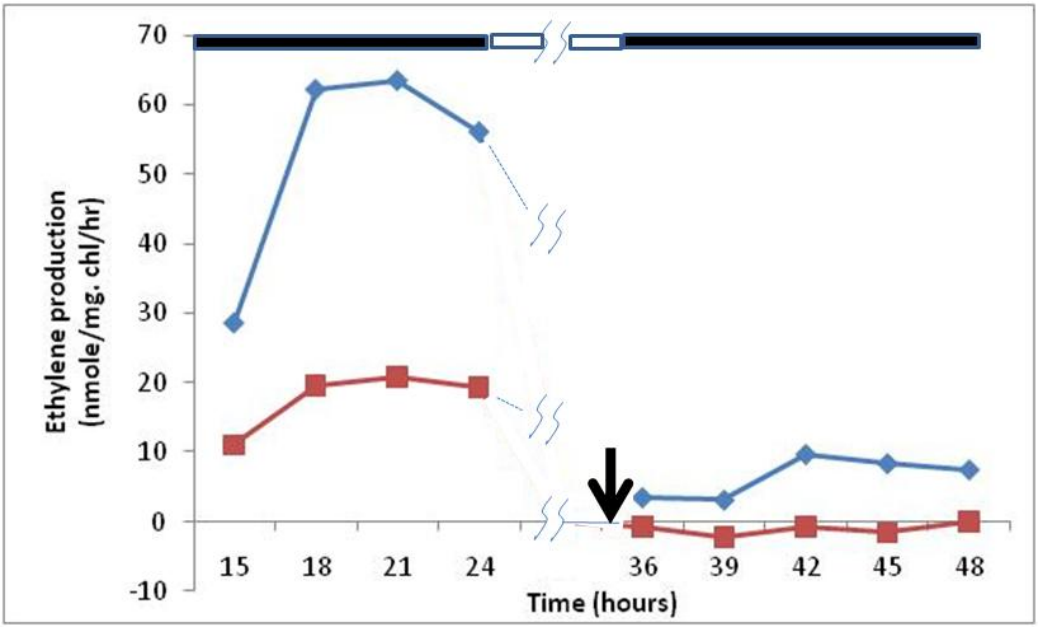

Fig. 27: Effect of chloramphenicol on dinitrogen fixation. Results are shown for two different experiments performed on different days, demonstrating that absolute rates vary, but the trend is the same in replicate experiment. Cultures were illuminated after 12 hours of light, then carried through an additional 12 h dark:12 h light:12 h dark. The first shown datum point (15 hours) is after 12 hours of light and 3 hours of dark. Chloramphenicol, at a concentration of 20 µg/mL, was added to cultures at the time indicated by the arrow.

In the presence of DCMU, which induces dinitrogenase activity in the light, the addition of chloramphenicol during darkness, one hour before a light period, has no detectable effect on dinitrogenase activity in the subsequent light period (Fig. 28). Thus, apparently, no new synthesis of dinitrognenase is required immediately prior to the light period, or during the light period, to initiate light-dependent activity.

Figures 27 and 28 together indicate that protein synthesis is required to initiate dinitrogenase activity in darkness following a photoperiod in

photosynthetically active cultures. However, no protein synthesis is required to activate dinitrogenase during the light period in cultures that have been inhibited by DCMU. Chloramphenicol is not water soluble and could only be added to the cultures in an organic solvent. Experiments shown in figures 27 and 28 used chloramphenicol added to cultures at a final ethanol concentration of 0.14%. Control experiments demonstrated that 0.14% ethanol had no effect on the pattern of ethylene production in the presence or absence of DCMU.

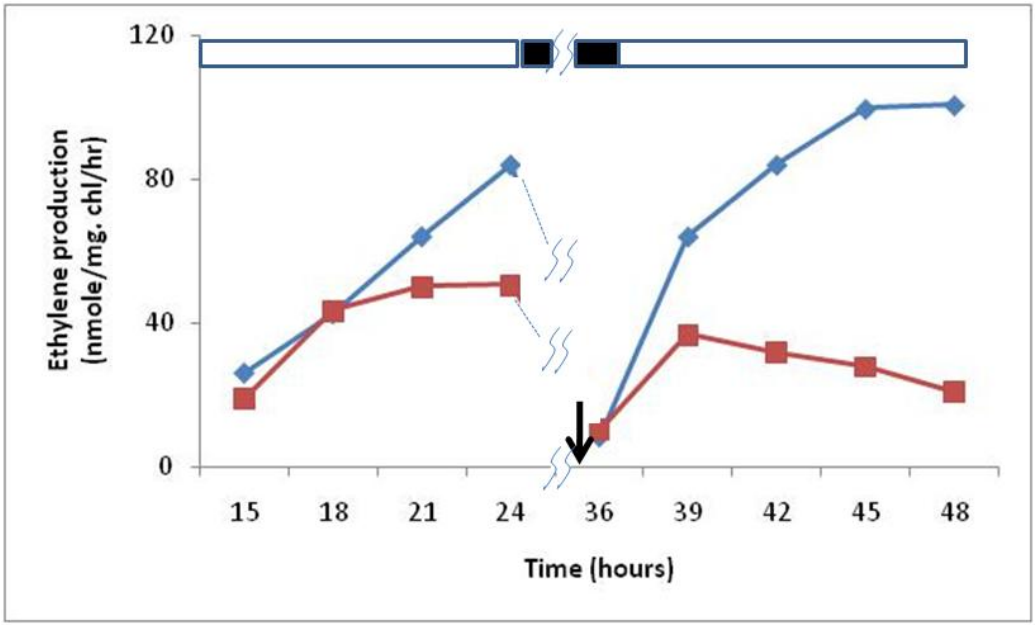

Fig. 28: Effect of chloramphenicol on dinitrogen fixation in the presence of 100 µM DCMU. Results are shown for two different experiments performed on different days, demonstrating that absolute rates vary, but the trend is the same in replicate experiment. DCMU was added at time zero, the beginning of a 12-hour dark period. The culture was illuminated after 12 hours of darkness, then carried through an additional 12 h light:12 h dark:12 h light. The first shown datum point (15 hours) is after 12 hours of darkness and 3 hours of light. Chloramphenicol, at a concentration of 20 µg/mL, was added to the culture at the time indicated by the arrow.

*7. nif*H gene in *L. nodulosa*

The dinitrogenase complex of all dinitrogen-fixing organisms is synthesized from DNA in an operon consisting of three structural genes designated as *nif*HDK (Bergman *et al.* 1997). Of these genes, *nif*H is most frequently used as a marker for the presence of dinitrogenase in an organism because it is relatively conserved compared to *nif*DK (*Zehr et al.* 1996). However, surprisingly little homology over long regions of the *nif*H gene occurs among various diazotrophs (Zehr *et al*. 1997). Degenerate primers have been developed that appear to selectively identify *nif*H in a broad range of dinitrogen-fixing organisms, including symbiotic and free living heterotrophic bacteria and cyanobacteria (Zehr & McReynolds 1989). This degenerate pair of primers (Appendix IV) was used to amplify a 338-nuceotide fragment (Appendix III) of extracted *L. nodulosa* DNA. Although this fragment did not amplify well and did not consistently produce a product, only one PCR product was seen in electrophoresis gels, which eventually was sequenced. The end sequences were used to synthesize specific primers (Appendix IV), which generated a much higher yield of PCR product.

The PCR product was cloned into a plasmid and inserted into *E. coli*. Plasmids from 5 clones of *E. coli* were sequenced and aligned. All sequences were identical except for single nucleotide differences in three positions close to each other (underlined in the *nif*H sequence shown in Appendix III). These very minor differences may be due to sequencing errors.

The sequence shown in Appendix III was compared to sequences in GenBank. BLAST results showed that all highly similar sequences have been identified as *nif*H genes. Phylogenetic analysis demonstrated that the most closely related sequences were from marine prokaryotes (Fig. 29). The sequence closest to that of *L. nodulosa* had 87% homology, relatively low for sequences less than 500 nucleotides in length. The sequences most similar to the putative *nif*H sequence in *L. nodulosa* were obtained from DNA extracted from unknown components of seawater (Moisander *et al*. 2007), so the morphological characteristics and taxonomic position of the strains that contain these sequences are unknown. The previously identified organism with a sequence most homologous to the putative *nif*H sequence in *L. nodulosa* is another strain of *Leptolybgya*. According to analysis shown in Fig. 29, the *Rhizobium* (a heterotrophic facultative symbiotic bacterium) sequence is more closely related to the sequence of *L. nodulosa* than is the sequence from *Trichodesmium* (a non-heterocystous cyanobacterium) or the sequence from *Nostoc* (a heterocystous cyanobacterium) (Fig. 29).

Although the same *nif*H gene (Appendix III) was amplified from both axenic and non-axenic cultures of *L. nodulosa*, ethylene production was only detected in non-axenic cultures. It remains to be explained why bacteria are required to facilitate dinitrogenase activity in *L. nodulosa*.

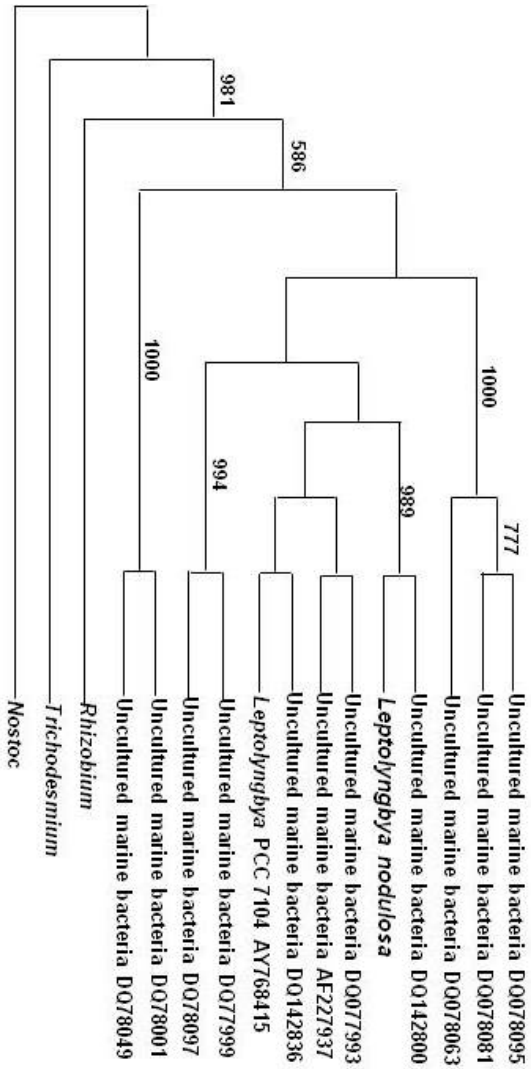

Fig. 29: Neighbor-Joining phylogenetic tree generated from *nif*H gene sequences. *Nostoc* was used as the outgroup. The number above each node is its bootstrap value (numbers less than 500 are not shown).

IV. DISCUSSION

A. The Genus *Leptolyngbya* and *L. nodulosa*

The genus *Leptolyngbya* was established within the Oscillatoriales to include species in “LPP Group B” in the system of Rippka *et al*. (1979), largely based on thin trichomes. Several genera with trichomes ≤ 3 μm have now been separated within this group of Oscillatoriales (Anagnostidis & Komárek 1988). *Leptolyngbya* was distinguished from other genera by the epiphytic mode of life in *Liebleinia* and the lack of a sheath in *Jaaginema* (Anagnostidis & Komárek 1988). However, characters used for classification of these related genera are influenced by culture conditions. For example, the formation and characters of sheaths are greatly affected by environmental conditions (Albertano & Kováčik 1994). Thus, morphological characters used for classification of *Leptolyngbya* are of questionable value and the genus *Leptolyngbya* remains problematic (Albertano 1991; Albertano & Kováčik 1992; Hoffman & Compere 1991). It seems that the establishment of the genus *Leptolyngbya* is a compromise that does not solve the problems posed by Rippka *et al*. (1979). Yet, non-heterocytous cyanobacteria with thin filaments appear to be almost ubiquitous in fresh waters, marine habitats and soils, displaying a great variety of subtle morphological variations (personal observations). Thus, based on morphology alone, the genus *Leptolyngbya* (*sensu lato*) may eventually be divided into a number of distinct genera.

Even the name of the type species is somewhat confused. A cyanobacterium identified by Gomont (1899) as *Plectonema boryanum* was transferred to a new genus (*Leptolyngbya*) by Anagnostidis and Komárek (1988) and re-described as the type species, *L. boryana*. However, strains referenced to that same type species are often designated as *L. boryanum*, based on the original *Plectonema* epithet. In fact, the Pasteur Culture Collection of Cyanobacteria lists two strains in their collection (PCC 73110 and PCC 6306) under the names *L. boryanum* and *L. boryana,* respectively.

Recent molecular phylogenetic analyses indicate that the genus *Leptolyngbya* should be revised. Using cold temperature strains, Casamatta *et al*. (2005) presented evidence that the genus *Leptolyngbya* should be divided into at least 3 genera. The phylogenetic analysis presented in this dissertation, based on 16s rDNA, also indicates that *Leptolyngbya* is not monophyletic (Li & Brand 2007). *L. nodulosa* occurs in a clade completely distinct from the clade containing the type species, but appears to be related to *Oscillatoria neglecta*. The latter species was re-named *Jaaginema neglecta* by Anagnostidis & Komárek (1988) in recognition of its thin filaments, but it is still recognized by its former name in many publications. The phylogenetic analysis of ITS sequences presented in this dissertation supports the 16s rDNA sequence analysis, indicating that *L. nodulosa* is closely related to strains identified as *Phormidium* and *Oscillatoria*. Therefore, the genus *Leptolyngbya* needs major revision to resolve problematic species. It seems likely that *L. nodulosa* will eventually be placed into a taxon

completely distinct of *L. boryana* (*L. boryanum*), perhaps establishing a new genus.

B. Morphological features of *L. nodulosa* and their potential functions in nature

Filaments of *L. nodulosa* cultures have several features which together distinguish it from other species of *Leptolyngbya*, including their flattened shape, a sheath of variable thickness, cells with an electron transparent space between the cell wall and the electron dense sheath, and especially the occurrence of nodules. These features likely all have ecological and habitat implications.

No filamentous cyanobacterium has previously been reported to have a flattened shape in cross section. This morphology provides a larger surface-to-volume ratio than would a round cross section, which may facilitate better light penetration at low light intensities. Since *L. nodulosa* was isolated at 10 m depth in eutrophic waters, it likely was exposed to very low light intensities. In laboratory cultures, *L. nodulosa* forms sheet-like structures and sometimes thick aggregates. The flattened morphology may be correlated with these aggregated forms. It is also possible that the flattened filaments are related to the ability to form nodules. Neither filamentous eukaryotic algae nor filamentous cyanobacteria typically form kinks or sharp bends along a continuous train of healthy cells within the filament, nor are they generally flattened in cross section. Tubular structures cannot be bent sharply without kinking or severe stretching.

Nodules contain portions of filaments that are sharply bent without obvious distortion or tearing of the filament. A flattened morphology may account for the ability of filaments to bend sharply without severe distortion or damage.

Dehydration steps in preparation for transmission electron microscopy often distort portions of cells and tissues, producing artifacts in the final images. Thus, it was of concern that the large electron transparent spaces between the cell wall and the electron dense sheath may result from dehydration prior to fixing and embedding the filaments in preparation for transmission electron microscopy. For that reason, a freeze-fixing process was used to immobilize the specimen prior to dehydration. This procedure (Method II in Materials and Methods) produced transmission electron microscopic images showing electron transparent spaces very similar to the spaces seen in samples prepared by the normal dehydration process (Method I of Materials and Methods). Thus, it appears that, at least in portions of a filament, the sheath is well separated from cell walls.

The function of the space under the electron dense sheath of *L. nodulosa* is not known, and may not be a feature of many cyanobacterial species. On occasion bacteria appear to be present under the sheath of a linear filament or within a nodule of *L. nodulosa*, although this observation was sporadic and not seen routinely in cultures that included bacteria. An example of apparent bacteria seen in light microscopy is shown in Fig. 30, and an example of a heterotrophic bacterium seen in a transmission electron micrograph of a nodule is shown in Fig.

31. The electron transparent area would provide sufficient space to harbor a typical bacterium. However, these spaces are not caused by heterotrophic bacteria since axenic and non-axenic cultures both demonstrate the feature. The space might also provide flexibility as cells divide and the trichome elongates, and when filaments bend sharply to form nodules, allowing the sheath to become thinner and drawn closer to the cell wall during expansion.

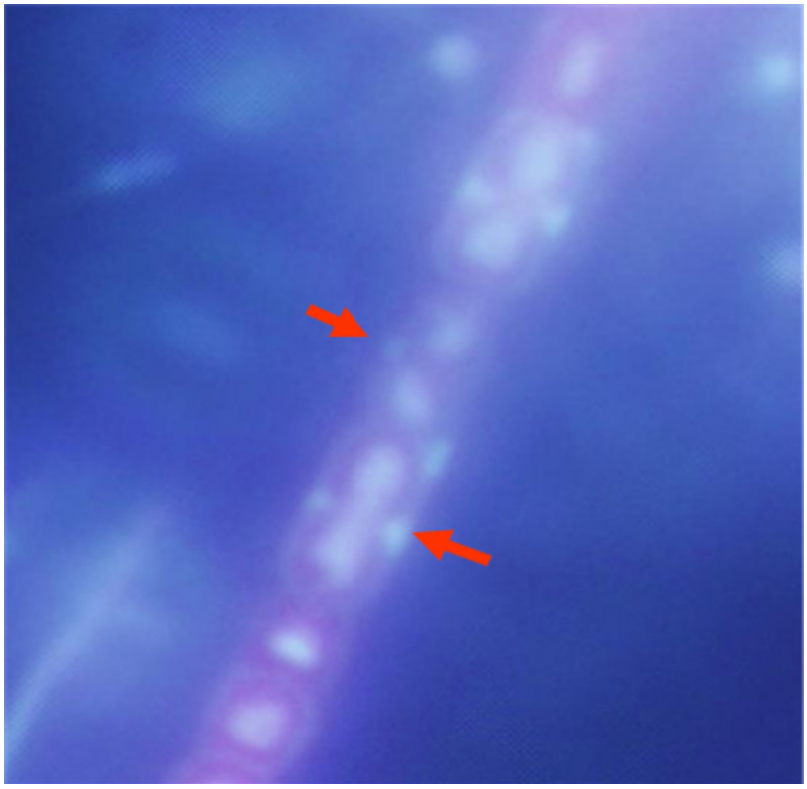

Fig. 30: DAPI-stained (blue color) image showing the DNA of cells within a trichome in a single row and putative bacteria (arrows) under the sheath of the filament. The red color is due to auto-fluorescence of chlorophyll.

Fig. 31: Transmission electron micrograph of a freeze-fixed sample of *L. nodulosa* showing a heterotrophic bacterium (arrow) within a nodule.

Hormogonia (with a very thin sheath) and mature filaments (with a well-defined sheath of variable thickness) were always both found in actively growing cultures. Necrotic sites were observed at various positions along mature filaments of these same cultures (Fig. 8). Often two or more necrotic sites were located within a few cells of each other along a trichome. These sites appear to serve as necridia (break points), releasing single cells or short filaments from the broken long filament, and releasing the short trichome from its sheath. Although

not all stages of this process have been observed in *L. nodulosa*, many observations suggest necridia may be an important means of reproduction. The active trembling of hormogonia may relate to the helical fibrils seen at the surface of the sheath (Hoiczyk 1998).

The unique feature of *L. nodulosa* is the occurrence of nodules. No definitive function of nodules has yet been demonstrated, although there are several reasonable possibilities.

a. Nodules may isolate portions of a filament within a micro-aerobic environment in order to facilitate dinitrogen fixation. The compact nature and continuous outer sheath of nodules support this possibility (Fig. 14). Also low light intensities, which are required for nodule formation, enhance anaerobic or micro-aerobic dinitrogen fixation during a subsequent dark period (Fig. 19). However, dinitrogen fixation occurs at approximately the same efficiency in cultures with or without nodules, and the ultrastructure of cells within nodules appears identical to that of cells in other portions of the filaments (Figs. 12 & 14). Therefore, nodules are not required for dinitrogen fixation in *L. nodulosa* cultures, and may perform photosynthesis as in other portions of the filament. It remains possible that conditions in nature favor dinitrogenase activity in nodules. *In situ* experiments using labeled antibodies against dinitrogenase may in the future provide a more definitive determination of sites along the filament where dinitrogenase is expressed.

b. Nodules may provide a favorable environment for bacteria that facilitate dinitrogen fixation. Dinitrogenase activity was not observed in axenic cultures; only cultures that were demonstrated to contain heterotrophic bacteria were capable of ethylene production. Also, nodules typically have electron transparent spaces between portions of a filament within the common sheath (Fig. 14), and heterotrophic bacteria may occupy this space (Fig. 31). However, axenic cultures of *L. nodulosa* contain the same *nif*H sequence as do cultures that also harbor heterotrophic bacteria, while no *nif*H sequence could be identified from heterotrophic bacteria isolated from non-axenic *L. nodulosa* cultures. Thus, it seems likely that a *nif*HDK operon is a component of *L. nodulosa*. It is still possible that a low concentration of an unculturable heterotrophic bacterium remained associated with “axenic” cultures, but were not detected by microscopic observations or culturing efforts.

These studies have not eliminated the possibility that a heterotrophic bacterial dinitrogenase facilitates dinitrogen fixation in cultures of *L. nodulosa*. It is possible that, because of the divergent nature of *nif*H genes in various dinitrogen-fixing organisms, these studies did not detect the *nif*H gene responsible for dinitrogenase synthesis in non-axenic cultures of *L. nodulosa*. The gene might then be a component of the cyanobacterium or of a symbiotic heterotrophic bacterium. Alternatively, bacteria might facilitate dinitrogen fixation in non-axenic cultures by providing a cofactor or other factor essential for the assimilation of N_2.

c. Nodules may protect *L. nodulsa* filaments from predation or other damage in nature. Some nodules increase the diameter of portions of a filament by an order of magnitude, thereby making predation by small zooplankton more difficult. The addition of a sheath around the entire nodule may provide a more robust barrier against predation. In nature nodules may protect portions of a filament against physical, chemical, and/or biological damage, since they resist disruption in laboratory cultures except under rather severe shear force.
d. Nodules could be buoyancy devices that regulate the depth of *L. nodulosa* filaments in the water column. However, filaments in cultures have a buoyant density almost identical to the density of MASM medium regardless of whether nodules are present or not, and microscopic observations did not reveal any gas vacuoles. Thus, unless the nodules of cultures in nature are somewhat different from those maintained in MASM, it seems unlikely that nodules provide buoyancy.

C. **Dinitrogen fixation in cultures of *L. nodulosa***

Non-axenic cultures of *L. nodulosa* fix dinitrogen in darkness under anaerobic and micro-oxygenic conditions, but not in the presence of even dim light or in atmospheric oxygen (Fig. 19). These requirements suggest that cultures contain a typical oxygen-sensitive dinitrogenase enzyme, which is readily inactivated by both ambient and photosynthetically-generated oxygen. The sensitivity of dintrogenase to low light intensities suggests that this enzyme

may be synthesized in photosynthetically active cells. Rates of respiration are high during dark periods between intervals of light, so active dinitrogenase may be synthesized very soon after the end of the photoperiod. Experiments described in this dissertation demonstrate high dinitrogenase activity within 3 hours after the beginning of a dark period. These observations, along with identification of the *nif*H gene in axenic cultures of *L. nodulosa*, suggest that dinitrogenase activity occurs within cells of *L. nodulosa* trichomes. However, dinitrogen-fixing bacteria under the sheath of filaments would also be directly exposed to photosynthetically-generated oxygen and might be exposed to ambient oxygen. An attractive, but unsubstantiated, model is a symbiotic system in which *L. nodulosa* provides an organic carbon source to heterotrophic bacteria embedded under the sheath, while the bacteria supply a source of combined nitrogen to the trichome.

In addition to its inhibitory effect on photosynthetic oxygen evolution, DCMU had profound effects on dinitrogen fixation as measured by acetylene reduction in cultures of *L. nodulosa*. The diurnal cycle of ethylene production was greatly altered in the presence of DCMU, such that most activity occurred in the light. The absolute rate of maximum activity was an order of magnitude higher in the presence of DCMU than in its absence. This photosynthetic inhibitor is highly selective in its inhibition of the Q_B site of Photosystem II (Van Reensen 1982), and has never been reported to directly affect the dinitrogenase enzyme

complex. Thus, it is reasonable to assume that inhibition of Photosystem II profoundly alters the regulation of dinitrogenase function.

The following model, illustrated in Figs. 32 & 33, is an effort to explain dinitrogenase activity in the presence and absence of DCMU. *L. nodulosa* performs photosynthesis while adjacent heterotrophic bacteria may perform dinitrogen fixation, although the model is applicable under the assumption that both photosynthesis and dinitrogen fixation occur within cells of *L. nodulosa*. The model assumes that ATP is generated via cyclic photophosphorylation in the presence or absence of DCMU (Ramanujam *et al*. 1981). This photosynthetically produced ATP, along with reduced carbon reserves, drives dinitrogenase activity.

Fig. 32 illustrates the relationship between photosynthesis and dinitrogen fixation in the absence of DCMU. Photosynthetically produced reductant (NADPH) and energy (ATP) are stored in the form of reduced organic substance in the light. The simultaneous production of molecular oxygen precludes dinitrogenase synthesis or activity. In the subsequent dark period, residual respiration quickly allows rapid regeneration of dinitrogenase. An anaerobic environment is quickly established in the subsequent dark period due to high respiratory activity (Fig. 23), so synthesis of dinitrogenase can begin almost immediately. The stored reduced organic substance that was produced during the preceding light period may be oxidized and broken down in this respiratory process to generate the reductant and ATP necessary for dinitrogenase activity. The potent inhibitory effect of chloramphenicol on the initiation of dinitrogenase

activity at the beginning of the dark period (Fig. 27), and the lack of sustained dinitrogenase activity over a long period of darkness (Fig. 21), supports this hypothesis.

The effect of DCMU on the diurnal cycle of dinitrogen fixation is illustrated in Fig. 33. Cyclic photophosphorylation produces the energy required for dinitrogenase activity, and reduced organic substance that was stored prior to the addition of DCMU produces reductant, facilitating dinitrogenase activity in the light. The model predicts that diurnal dinitrogenase activity can only be sustained until internal reserves of reductant are exhausted. Future experiments will test that possibility.

The work presented in this dissertation demonstrates that cyanobacteria are morphologically more diverse than previously thought. It also identifies a new organism that may contribute significantly to the nitrogen balance of tropical/subtropical oceans. I hope to address the following important unanswered questions regarding this organism in the near future.

1. What is the physiological role of nodules in *Leptolyngbya nodulosa*?
2. What roles do heterotrophic bacteria play in facilitating dinitrogenase activity in *Leptolyngbya nodulosa*?
3. How prevalent is *Leptolyngbya nodulosa* in nature and what is its ecological significance in nitrogen fixation?

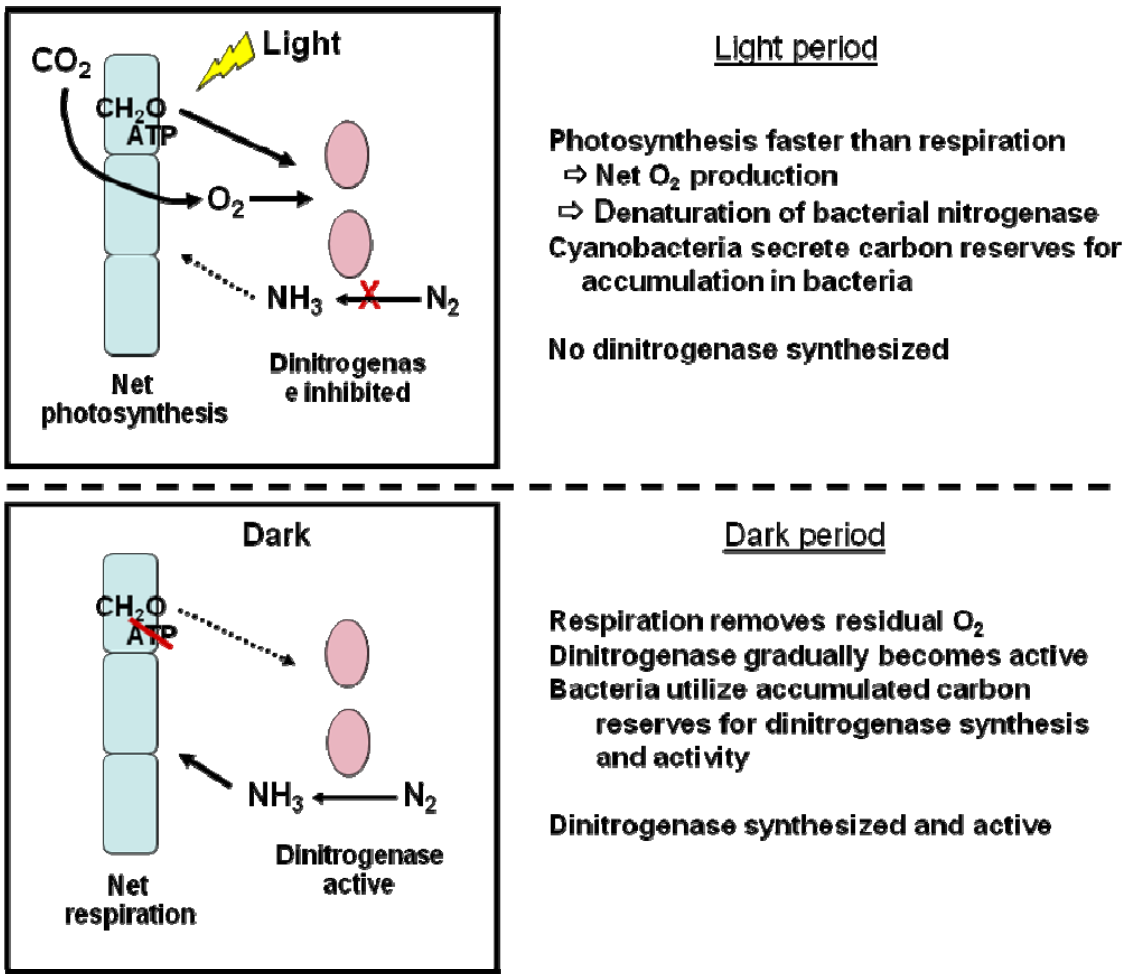

Fig. 32: Model of dinitrogenase activity in a symbiotic system including *L. nodulosa* and a dinitrogen-fixing heterotrophic bacterium.

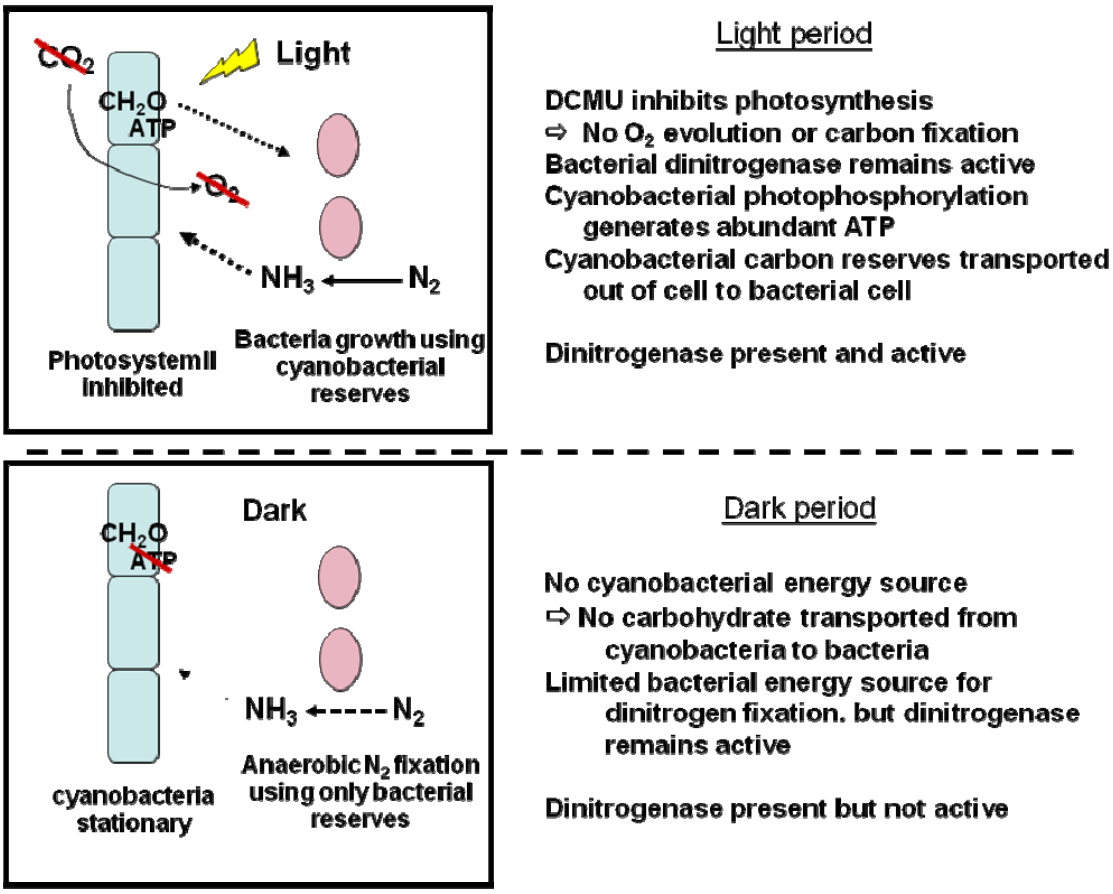

Fig. 33: Model of dinitrogenase activity in a symbiotic system including *L. nodulosa* and a dinitrogen-fixing heterotrophic bacterium in the presence of DCMU.

Appendix I

Table A1: Recipe of Modified Artificial Seawater Medium (MASM)

Compound	Concentration (mg/L)	Concentration (mM)	Reference
NaCl	30000	512.8	
$MgSO_4.7H_2O$	2440	10	
KCl	600	8.0	
$NaNO_3$	1000	11.8	
$CaCl_2.2H_2O$	300	2.2	
KH_2PO_4	50	0.4	
Tricine	1000	5.6	
NH_4Cl	27	0.5	
P-IV trace elements	1 mL/L	N/A	Starr and Zeikus 1993
Soilwater medium (GR+)	30 mL/L	N/A	Starr and Zeikus 1993
Vitamin B_{12}	1 mL/L	N/A	Starr and Zeikus 1993
Thiamine	1 mL/L	N/A	Starr and Zeikus 1993
Biotin	1 mL/L	N/A	Starr and Zeikus 1993

Components were added in the order listed above. The medium devoid of vitamins was adjusted to pH 8.0 with NaOH and autoclaved. The last three components were then added aseptically.

MASM-0

MASM-0 was prepared exactly the same as was MASM, except that $NaNO_3$, NH_4Cl and soilwater medium were not included, and the volume of added P-IV trace elements was 6 mL.

Both MASM and MASM-0 were stored at 4°C prior to use.

Appendix II

Tolerance of Various Cyanobacteria to Different Salinities

This set of experiments was conducted to determine if the ability of *L. nodulosa* to grow in media of widely varying salinities is commonplace among cyanobacteria. A fresh inoculum of each strain to be tested was first inoculated into its normal growth medium and allowed to grow for two weeks under normal culture conditions. Each of these recently grown cultures was then made homogeneous by insertion into a sonic bath for 30 seconds at ambient temperature. This process did not kill the cyanobacteria but disrupted clumps and substantially shortened the lengths of filaments. The dispersed culture was divided into equal aliquots and each aliquot was subjected to gentle centrifugation (just sufficient to pellet most of the cyanobacteria). Each pellet was suspended in a test medium (Table A2) and the OD_{678} was determined as the measure of initial culture density. Initial densities were just high enough to be measured reliably in the spectrophotometer. A thirty-ml volume of each culture was left undisturbed under conditions expected to promote growth (temperature 25 C, illumination 60 µmol photons m^{-2} sec^{-1}, 12 h light:12 h dark diurnal cycle) After 21 days of incubation time each culture was again subjected to gentle sonication and the OD_{678} was determined as a measure of final culture density. Culture media that supported at least a doubling of the OD_{678} value over the 21-day growth period were considered to be supportive of growth (Table A3). An

asterisk indicates that growth was supported; a minus sign indicates that growth was not supported.

Table A2: Media used in these experiments

Media	Test medium	Modification	Reference
VM	Freshwater	none	Starr and Zeikus 1993
BG-11	Freshwater	none	Rippka et al. 1979
BG-11+	Saline	3% NaCl added	
BG-11++	Hypersaline	6% NaCl added	
MASM+	Saline	none	Appendix I
MASM++	Hypersaline	3% NaCl added	

Table A3: Growth of cyanobacteria in different media

Strain	Habitat	Morphology	Medium					
			VM	BG-11	BG-11+	BG-11++	MASM	MASM+
Leptolyngbya nodulosa UTEX2910	Marine	Filamentous, nonheterocystous	*	*	*	*	*	*
Synechococcus sp UTEX2380	Marine	Unicellular	*	*	*	*	*	*
Spirulina platensis UTEX1926	Marine	Filamentous, nonheterocystous	*	*	*	-	*	*
Lyngbya aestuarii UTEX2515	Marine	Filamentous, nonheterocystous	-	-	-	-	*	*
Anabaena sp UTEX2497	Marine	Filamentous, heterocystous	*	*	*	*	*	*
Anabaena verrucosa UTEX1629	Freshwater	Filamentous, heterocystous	-	*	-	-	*	-
Nostoc punctiforme UTEX1619	Freshwater	Filamentous, heterocystous	-	*	-	-	*	-

Appendix III

Sequences of the Genes Identified from *L. nodulosa*

1. 16s rDNA (partial)

AGTCGAACGGACCCTTCGGGGTTAGTGGCGGACGGGTGAGTAACG
CGTGAGGATCTGCCCTTAGGAGGGGAACAACAGTTGGAAACGACTG
CTAATGCCCCATATGCCGAGAGGTGAAAAGTTATATCGCCTGAGGAT
GAACTCGCGTCTGATTAGCTAGTTGGTAGGGTAAAGGCTTACCAAG
GCGACGATCAGTAGCTGGTCTAAGAGGATGATCAGCCACACTGGGA
CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTT
TCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGGGAG
GAAGGCCTTAGGGTTGTAAACCTCTTTTCTCTGGGAAGAAGAACTGA
CGGTACCAGAGGAATAAGCCTCGGCTAACTCCGTGCCAGCAGCCGC
GGTAAGACGGAGGAGGCAAGCGTTATCCGGAATTATTGGGCGTAAA
GCGTCCGCAGGCGGCTAATTAAGTCTGTTGTCAAAGGTCACAGCTC
AACTGTGGATCGGCAATGGAAACTGGTTAGCTAGAGTGTGGTAGGG
GTAGAGGGAATTCCCGGTGTAGCGGTGAAATGCGTAGATATCGGGA
AGAACACCAGTGGCGAAGGCGCTCTACTGGGCCACAACTGACGCTG
AGGGACGAAAGCTAGGGGAGCGAAAGGGATTAGATACCCCTGTAGT
CCTAGCTGTAAACGATGGATACTAGGTGTTGGACGTATCGACCCGT
GCAGTACCGTAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGC
ACGCAAGTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCG
GTGGAGGATGTGNNTTAATTCGATGCAACGCGAAGAACCTTACCA

2. Internal transcribed spacer (ITS) (complete), including two inserts, $tRNA^{Ile}$ (red color) and $tRNA^{Ala}$ (blue color).

TAGGGAGGGTTATCCTTGATAAGTCTGAGCCTCGGTGATTTAGGAGT
ATTGAGTGGTCGATTTAGATCGCTCAGGTTTGTGAGTAGAGTTTCTG
GACTAGGGTTGAGGTTTATTTGTCGCCTAAGCATCCGGTTAGAGAAG
GGAAGAGGACCAACACGCGGTGTACTATCTCGGGCTGGTTAGAGGG
CACCCCTGATAAGGGAGAGGTGCGTGGTACGAGCCCGGGATGATA
CACTGGGTGGGGTTCTAGTTCAGTTGGTAGAGCGCGTATTAGCAAG
CAGAATGTCATCGGTTAGAGTCAGATAACCTCCTCCACGAAAGCATG
GGATTGAATTCAGCCACGGCTGTGTTTTGGTGATAAGCAAGGCATAG
TGTTATTGGGTGAGAGCTTAGCAAAGAGCAGGAAACCAAATACGCG
AGAAGTGTAAGGTAGTACTCACAGATCATTCACAGTTCGTTGAGAGA
ACGAGTGAACTTGATCTAGTTTTGATTAGGTAATCGAGAGAA

3. *nif*H gene (partial)

CCACGTTCTATGCTGCCAGCNAAGGCCCAACCACGTTTTCTGCACCT
AGCGGCTGAGCGCGGTGCCGTTGAGGACCTAGAGCTAGATGAAGT
GCTGCTCAAGGGCTACAAAGACGTCAAGTGCGTTGAGTCCGGTGGT
CCTGAGCCAGGAGTTGGCTGTGCCGGTCGAGGCATTATCACCGCGA
TTAACTTCCTAGAGGAAGAAGGCGCTTACGAAGATCTCGACTTTGTT
TCCTACGACGTATTGGGCGACGTGGTTTGCGGCGGTTTTGCCATGC
CGATCCGGGAAGGTAAAGCCCAGGAAATCTACATCGTCTGCTCCGG
TGAAATGATGGCCCT

Appendix IV

Primers Used in the Experiments Described in This Dissertation

Primers for amplification of 16s rDNA in *L. nodulosa*

Forward: 5'-AGA GTT TGA TCC TGG CTC AG-3'

Reverse: 5'-ACGCCGACCTAGTGGAGGAA-3'

Primers for amplification of ITS sequence in *L. nodulosa*

Forward: 5'-CTC TGT GTG CCT AGG TAT CC-3'

Reverse: 5'-TGT ACA CAC CGG CCC GTC-3'

Degenerate primers for amplification of *nif*H gene in *L. nodulosa*

Forward: 5'-TGYGAYCCNAARGCNGA-3'

Reverse: 5'-ADNGCCATCATYTCNCC-3'

Note: Y = C, T
N = A, C, T, G
R = A, G;
D = A, G, T

Specific primers developed for identifying the *nif*H gene in *L. nodulosa*

Forward: 5'-AGGGCCATCATTTCACC-3'

Reverse: 5'-CTAGCGGCTGAGCGCGGTGC-3'

Appendix V

Calculations of Ethylene Production

Acetylene gas was prepared by adding ~ 5 mL of water to ~10 g of calcium carbide through a serum cap in a closed flask. The generated gas was trapped in a gas sample bulb until use. Regent grade ethylene gas at 1,000 ppm was purchased from Matheson Tri-Gas.

A standard gas mixture was prepared by adding 4.0 mL of acetylene and 1.0 mL of ethylene through a serum cap to a 25-mL reaction flask containing 5.0 mL of MASM-0. The concentration of ethylene in the flask is then 0.22 μmole/L. A 100-μL volume of this gas mixture was injected into the gas chromatograph. Peak areas of ethylene “E” (retention time = ~ 1.5 minutes) and acetylene “A” (retention time = ~ 3.0 minutes) were measured. The ratio E/A was calculated and recorded as $(E/A)_S$.

The method of preparing sample for acetylene reduction assays is described in Materials and Methods, and a sample calculation for determining rates of acetylene reduction is shown on the following page.

The amount of culture was measured as wet weight (see Materials and Methods) and converted to chlorophyll content using the conversion ratio of 120 μg chlorophyll = 1 g wet weight. In multiple measurements of 4 different samples, this ratio varied by ~25%.

Sample Calculation of Rate of Acetylene Reduction
(Data from March 1st, 2007)

Conversion factor: 123 µg chl a = 1 g wet weight.

Wet weight of sample: 0.79 g

Standard acetylene/ethylene ratio:

A gas mixture containing 1 mL (1000ppm) of standard ethylene and 4 mL of pure acetylene was placed into a 25-mL flask containing 5 mL MASM-0 medium. The flask then contained 0.223 nmoles/mL of ethylene. One hundred microliters of this mixture was injected into the GC.

GC data: Standard gas: Acetylene peak area = 276321
Ethylene peak area = 73.31559

Sample:

At time 0 Acetylene peak area = 252777
Ethylene peak area = 2.6628

At time 3 hours Acetylene area = 262295
Ethylene area read = 11.34453

Calculations

Ratio of ethylene to acetylene peak areas in standard gas mixture (R_s)
= 73.31559/276321 = 0.000265328

Ratio of ethylene to acetylene peak areas in sample:
At time zero (R_0) = 2.6628/252777 = 0.000010534
At time 3 hours (R_3) = 11.34453/262295 = 0.000043251

Absolute ethylene (E)
$E_0 = R_0/0.000265328$ x 0.223 nmole/mL x 20.0 mL = 0.177 nmole
$E_3 = R_3/0.000265328$ x 0.223 nmole/mL x 20.0 mL = 0.728 nmole

Ethylene production rate (nmole/mg. chl/hr)
$= (E_3 - E_0)/(T_3 - T_0)$/chl a = (0.728-0.177) nmole/(3-0) hr/(123 µg/g x 0.79g)
= 0.00194 nmole/µg. chl/hr
= 1.94 nmole/mg. chl a/h

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VITA

Zhongkui Li was born in Xinzhuang, Feixian, Shandong Province, P.R. China on October 12th, 1963, the son of Jinglan Li and Chuanyu Guo. After completing his work at the First High School of Feixian, Shandong Province, he entered Shandong Normal University in Jinan, Shandong in 1982. He received the degree of Bachelor of Science from the University in July 1986. During the following two years, he was a biology teacher at Experimental High School of Feixian in Shandong Province. In September 1988 he entered graduate school at Wuhan Institute of Botany, Chinese Academy of Sciences in Wuhan, Hubei Province. He received a degree of Master of Science from the Institute, in July 1991. He was then employed as a Research Assistant, Assistant Professor and Associate Professor at the Wuhan Institute of Botany, Chinese Academy of Sciences. In September 2002 he entered the Graduate School of the University of Texas at Austin.

Permanent Address: Wuhan Institute of Botany, Chinese Academy of Sciences, Wuhan, 430074, Hubei Province, P.R. China

This dissertation was typed by the author.